







THE JOURNAL OF  
GENERAL PHYSIOLOGY





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THE JOURNAL  
OF  
GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB

W. J. V. OSTERHOUT

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## CONDUCTIVITY AND PERMEABILITY.

By W. J. V. OSTERHOUT.

*(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)*

(Received for publication, July 19, 1921.)

When an electrical current passes from a salt solution into a living cell, ions must enter the protoplasm. An increase in the permeability of the protoplasm to ions must decrease its electrical resistance, and *vice versa*. The electrical resistance of the protoplasm may therefore be regarded as a measure of its permeability to ions.

If we attempt to measure the electrical resistance of the protoplasm we must first consider the structure of the tissue. For example, we find in the case of *Laminaria* that the protoplasm of each cell forms a thin layer which surrounds a large central vacuole filled with cell sap. Since experiments have shown that the cell sap has about the same electrical resistance as the solution which bathes the cell, it is evident that when the electrical resistance of the cell increases, on transferring it from sea water to another solution of the same conductivity, the change must be due to an increase in the resistance of the thin layer of protoplasm which bounds the cell. This has led the writer to assume that the resistance is proportional to a substance, M, at the surface of the cell; if M forms a layer at the surface it is obvious that an increase in the thickness of this layer will increase the resistance, and *vice versa*. It is therefore assumed that the resistance depends upon the amount of M which is present in the surface.<sup>1</sup>

In *Laminaria* the protoplasmic masses (cells) are separated from each other by a thin layer of gelatinous substance (cell wall). In passing through the tissue a part of the current goes through the protoplasm and another part passes between the protoplasmic masses,

<sup>1</sup> This assumption is simple and facilitates quantitative treatment. It is recognized that changes in resistance might depend upon other properties of this layer, and that the layer need not necessarily be continuous.

in the substance of the cell wall.<sup>2</sup> Consequently when we employ the electrical method we must ascertain whether we are investigating the permeability of the protoplasm or merely that of the cell wall.

Obviously the best method of attacking this problem is to kill the tissue by such means (*e.g.*, partial drying, heating to 35°C., weak alcohol, etc.) as can not alter the cell wall, and then investigate its behavior under the influence of various reagents. We find that all of these methods produce the same result. After death the tissue no longer shows the changes in resistance which are observed when living tissue is subjected to the influence of reagents. It is therefore evident that the changes are due to the living protoplasm.

The cell wall appears in all cases to have practically the same conductivity as the surrounding solution. If we subject living tissue to solutions of the same conductivity, but of different chemical composition, the resistance of the cell wall remains unaltered while that of the protoplasm undergoes great variations. If, for example, living tissue is placed in a solution of NaCl or CaCl<sub>2</sub> (of the same conductivity as sea water) its behavior differs. In NaCl the resistance falls; in CaCl<sub>2</sub> it rapidly rises and later falls to a minimum. We infer that the permeability of the protoplasm increases in NaCl; and that in CaCl<sub>2</sub> there is a decrease followed by an increase.

This is in complete agreement with results obtained when permeability is measured by such methods as plasmolysis,<sup>3</sup> specific gravity,<sup>4</sup> tissue tension, exosmosis, and diffusion through living tissue.<sup>5</sup> This agreement indicates that the electrical method measures the permeability of the protoplasm. It is however desirable to go further, if possible, and analyze the factors involved in electrical resistance.

<sup>2</sup> As explained in a former paper (Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 485) the fact that a part of the current passes through the protoplasm is shown by the fact that CaCl<sub>2</sub> raises the resistance of living tissue and by the fact that the temperature coefficient of electrical conductivity differs in living and dead tissue.

<sup>3</sup> Osterhout, W. J. V., *Science*, N. S., 1911, xxxiv, 187.

<sup>4</sup> Loeb, J., *Science*, N. S., 1912, xxxvi, 637. *Biochem. Z.*, 1912, xvii, 127.

<sup>5</sup> Brooks, S. C., *Proc. Nat. Acad. Sc.*, 1916, ii, 569. For exosmosis of the pigment of *Rhodymenia* in relation to electrical resistance see Osterhout, W. J. V., *J. Gen. Physiol.*, 1919, i, 299.



If we consider the behavior of the current from this point of view, it is evident that in the simplest cases, where the plant is a membrane only one cell thick (as in *Porphyra* and *Monostroma*) and the current passes through this membrane at right angles to its surface, we need consider only a single cell and its adjacent cell wall, as shown in Fig. 1, *A*. The part of the current which goes through the protoplasm may be designated as  $C_P$ , while that which traverses the cell may be called  $C_W$ .

Experiments show that the resistance of the living tissue is much greater than that of tissue which has been carefully killed with all possible precautions to prevent any alteration of the cell wall.<sup>6</sup> We therefore feel confident that the conductivity of the living protoplasm is less than that of the cell wall.

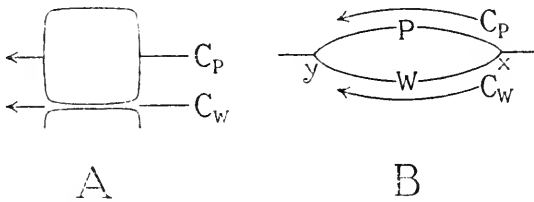


FIG. 1.

In order to see how the current may distribute itself let us suppose the protoplasm to be replaced by a wire,<sup>7</sup>  $P$ , as in Fig. 1, *B* and the cell wall to be replaced by a wire,  $W$ . The current flowing between

<sup>6</sup> Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1919, i, 299. *J. Biol. Chem.*, 1918, xxxvi, 485.

<sup>7</sup> We might consider the protoplasm to be replaced by two wires one of which corresponds to the thin layers of protoplasm which are traversed by the current in a direction at right angles to their planes, the other corresponding to the similar layers of protoplasm in each cell (around the edges of the cell shown in Fig. 1, *A*) in which the current flows in the plane of the layer. It is evident, however, that these latter may be neglected in our calculations since they occupy such exceedingly small fractions of the cross-section.

¶ If we neglect these we may say that in traversing a cell the current passes through a thin layer of cell wall and then one of protoplasm (in both cases at right angles to the plane of the layer), then through the cell sap, and finally through a layer of cell wall and one of protoplasm (at right angles to their planes). It is evident that in this case we may neglect the effect of the cell wall and of the cell sap since their resistance is very small in comparison with

the points  $X$  and  $Y$  in the wire  $P$  may be called  $C_P$ ; that in the other wire  $C_W$ . The total current,  $C$ , flowing between  $X$  and  $Y$  will be the sum of the partial currents, or,

$$C = C_P + C_W$$

We may consider the current (conductance) as equal to the reciprocal of the resistance and write

$$\frac{1}{R} = \frac{1}{R_P} + \frac{1}{R_W}$$

in which  $R$  is the total resistance between  $X$  and  $Y$ ,  $R_P$  is the resistance of the wire  $P$ , and  $R_W$ , that of  $W$ . Applying this equation to *Laminaria*<sup>8</sup> (and expressing the resistance in the usual way as the per cent of the normal) we may calculate the values of  $C_W$ ,  $C_P$ ,  $R_W$ , and  $R_P$ .

Under normal conditions in sea water, the resistance is taken as 100 and therefore  $C = 1 \div 100$  but in certain solutions (having the same conductivity as sea water) the resistance may rise to 300 or more; and in this case  $C$  would equal  $1 \div 300 = .0033$  (or less), and since some of it must flow in the protoplasm the amount which traverses the cell wall must be less than this. We are therefore safe in putting it as low as  $1 \div 350 = .002857$ .

All the experiments hitherto made indicate that the conductivity of cell the wall remains unaltered in spite of changes in the chemical

that of the protoplasm and is in series with it. We may therefore consider the protoplasm to be replaced by a single wire having a resistance equal to that of the two layers of protoplasm which are traversed by the current in a direction at right angles to their planes.

<sup>8</sup> So far we have considered only the simplest case, when the plant is only one cell thick. But it is evident that these considerations also apply when several membranes are placed together, forming a mass comparable to the tissue of *Laminaria*. The only difference is in that case the current would traverse a very thin layer of cell wall in passing from one protoplasmic mass to the next, so that what we have spoken of as the resistance of the protoplasm would be composed in part of the resistance of these cell walls. When the protoplasm is dead the total resistance is only 10.29 and the resistance of these cell walls must be only a small fraction of this. Consequently their resistance in the living tissue of *Laminaria* is undoubtedly less than 1 when that of the protoplasm is 140. The resistance of these cell walls may therefore be neglected.

character of the solution, provided the conductivity of the solution remains the same. We may therefore take .002857 as the fixed value of  $C_W$ .

Let us now consider what values  $C_P$  assumes as the resistance changes. In sea water we have<sup>9</sup>  $R = 100$  and

$$C = \frac{1}{100} = .002857 + C_P$$

whence  $C_P = .007143$  and  $R_P = 1 \div C_P = 140$ . In the same manner we find that when  $R = 90$ ,  $R_P = 121.15$ , and when  $R = 10$ ,  $R_P = 10.29$ .

The changes in resistance thus far discussed have been treated as though they occurred in sea water; in this case the experiments indicate that the conductivity of the cell sap remains practically constant and hence need not be taken into account in our calculations. We may now ask whether this is also the case when the changes in resistance occur in other solutions. In order to investigate this, experiments were made with solutions of NaCl and CaCl<sub>2</sub> (of the same conductivity as sea water). The tissue was placed in these solutions and removed after various intervals of exposure. It was cut into small bits and ground (so as to open the cells) and the conductivity of the expressed juice was compared with that of sea water. As no significant difference was found we may consider that the conductivity of the cell sap does not change sufficiently in these solutions to alter our calculations.

Let us now consider the changes in protoplasmic resistance which occur in toxic solutions. When tissue is placed in NaCl 0.52 M the net resistance falls rapidly. The death curve may be obtained by means of the formula<sup>10</sup>

$$\text{Resistance} = 2700 \left( \frac{K_A}{K_M - K_A} \right) \left( e^{-K_A T} - e^{-K_M T} \right) + 90e^{-K_M T} + 10$$

<sup>9</sup> The total conductance of the protoplasm is greater than that of the cell walls, but the protoplasm occupies a much greater fraction of the conducting cross-section than the cell walls, so that the actual conductivity of the protoplasm is much less than that of the cell wall.

<sup>10</sup> For the explanation of this formula see Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533; *J. Gen. Physiol.* 1920-21, iii, 145, 415, 611.

in which  $T$  is the time of exposure,  $K_A$  and  $K_M$  are constants, and  $e$  is the basis of natural logarithms. We find by means of this formula that in a solution<sup>11</sup> of NaCl 0.52 M (for which  $K_A = .018$  and  $K_M = .540$ ) the net resistance after 10 minutes is 87.76 per cent of the normal; after 30 minutes it is 64.26, and after 60 minutes it is 41.62. Knowing the net resistance we can calculate the protoplasmic resistance, as explained above. After 10 minutes the protoplasmic resistance is 117.12 per cent (corresponding to the net resistance of 87.76 per cent). Since it is desirable to express all resistances as per cent of the resistance in sea water we divide 117.12 by 140 (which is the protoplasmic resistance in sea water) and obtain 83.66 per cent. Proceeding in this way we find that after 30 minutes the

TABLE I.

*Velocity Constants at 15°C.*

CaCl <sub>2</sub> in solution.	CaCl <sub>2</sub> in surface.	$K_A$	$K_M$	$K_{AP}$	$K_{MP}$
<i>per cent</i>	<i>per cent</i>				
0	0	0.018	0.540	0.0234	0.702
1.41	12.5	0.000222	0.00666	0.000293	0.00878
2.44	20.0	0.000187	0.00546	0.000237	0.00708
4.76	33.33	0.000245	0.00590	0.00032	0.007136
15.0	63.73	0.000364	0.0073	0.0005035	0.00855
35.0	84.34	0.000481	0.00859	0.000678	0.00955
62.0	94.22	0.00053	0.009	0.000761	0.00989
100.0	100.0	0.0018	0.0295	0.002685	0.0323

protoplasmic resistance is 56.22 per cent and after 60 minutes 33.74 per cent. In order to fit the formula to these values we must change the constants, putting  $K_{AP} = 0.0234$  (in place of  $K_A = 0.018$ ) and  $K_{MP} = 0.702$  (in place of  $K_M = 0.54$ ). It is therefore evident that in changing from net resistance to protoplasmic resistance we merely shift the value of the constants. The question arises whether this affects the general conclusions drawn from the study of net resistance. In order to decide this question the constants for CaCl<sub>2</sub> and

<sup>11</sup> See Osterhout, W. J. V., *J. Biol. Chem.*, 1917, xxxi, 585.

for various mixtures of NaCl and CaCl<sub>2</sub> were ascertained; these are given in Table I.<sup>12</sup>

There are two points of principal importance in the consideration of these constants: (1) It was shown in a former paper<sup>13</sup> (which dealt with net resistance only) that the value of  $K_A \div K_M$  increases regularly as the per cent of CaCl<sub>2</sub> in the surface of the cell increases. That this is also true in the case of protoplasmic resistance is evident from Fig. 2. (2) It was also pointed out that as the per cent of CaCl<sub>2</sub> in the solution decreases from 62 to 1.41 per cent the value of  $K_M$  first decreases (reaching a minimum at 4.76 per cent) and then increases. It was found that the amount of decrease corresponds to

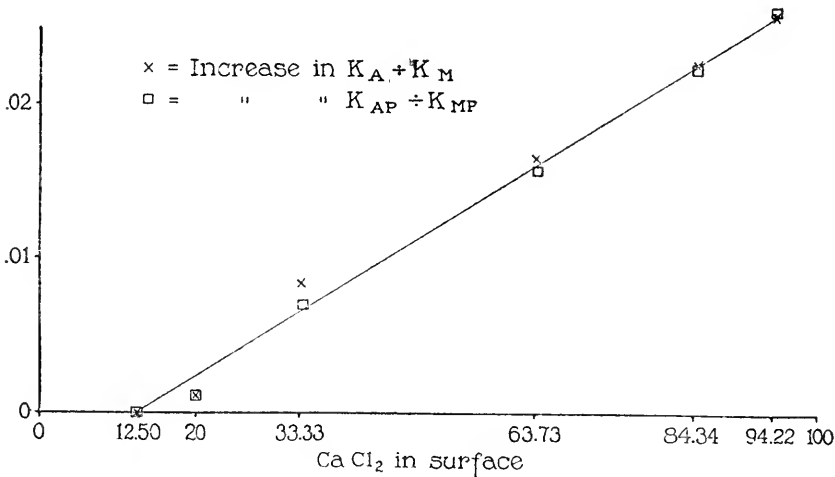


FIG. 2. Ordinates represent the increase in value of  $K_A \div K_M$  and of  $K_{AP} \div K_{MP}$ . In each case the value given represents the increase over the corresponding value in the solution containing 1.41 per cent CaCl<sub>2</sub> (the corresponding per cent in the surface being 12.5). Abscissæ represent per cent of CaCl<sub>2</sub> in the surface. In order to facilitate comparison the values of  $K_{AP} \div K_{MP}$  have been divided by 1.685.

<sup>12</sup> These are approximate values, obtained graphically. The constants of the curves of protoplasmic resistance are designated as  $K_{AP}$  (corresponding to  $K_A$ ) and  $K_{MP}$  (corresponding to  $K_M$ ). The curves of protoplasmic resistance may show less inhibition at the start than those of net resistance.

<sup>13</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1920-21, iii, 415.

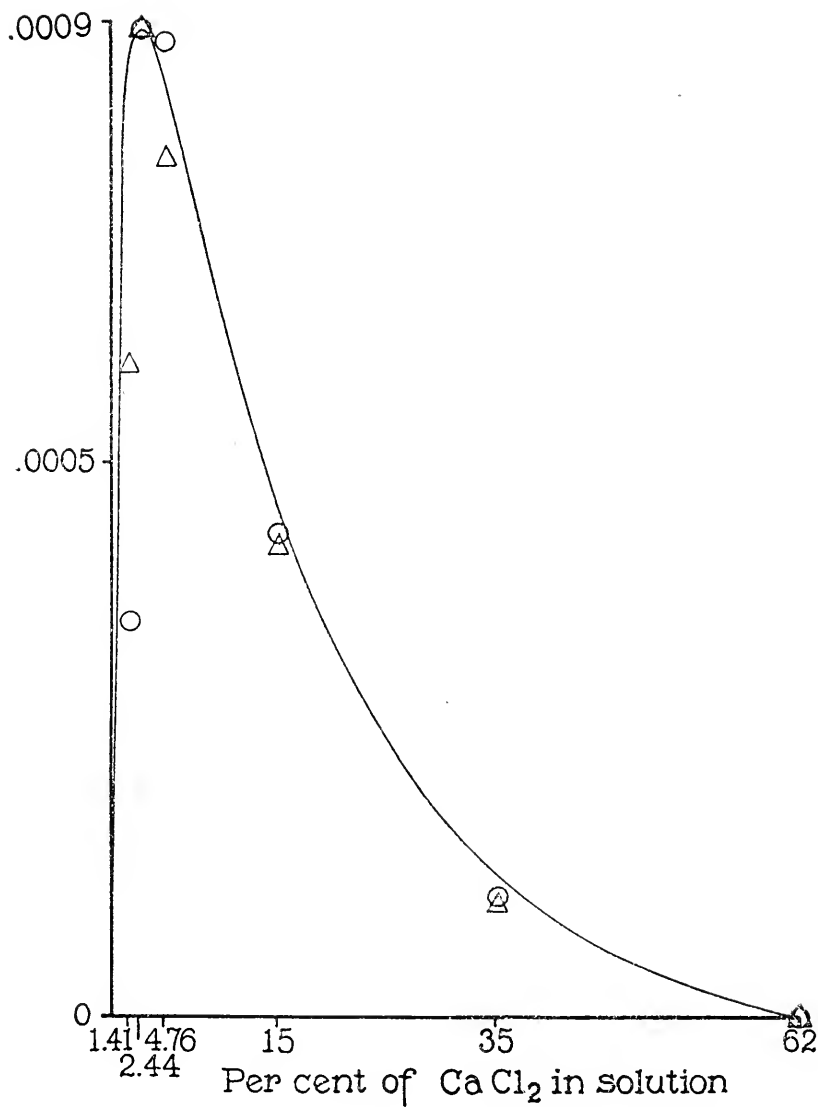


FIG. 3. Ordinates represent the amount of  $\text{Na}_4\text{XCa}$  and also the decrease in the value of  $K_M(\Delta)$  and of  $K_{MP}(\circ)$  as compared with the corresponding value in the solution containing 62 per cent  $\text{CaCl}_2$ . Abscissae represent per cent of  $\text{CaCl}_2$  in the solution. In order to facilitate comparison the values of  $K_M$  have been multiplied by 0.251 and those of  $K_{MP}$  by 0.321.

the amount of a hypothetical salt compound ( $\text{Na}_4\text{XCa}$ ). This is also true in the case of protoplasmic resistance, as shown in Fig. 3.<sup>14</sup>

It would therefore appear that we arrive at the same conclusions whether we study net resistance or protoplasmic resistance. When the solution is changed the constants change in a corresponding manner in both cases, the only difference being in their absolute values, but it is evident that in this case differences in absolute values are of no importance.

It should be emphasized that this general conclusion would remain valid in case it should be found that the values given in this paper for  $C_P$  and  $C_W$  are incorrect. There seems to be no doubt that the value of  $C_W$  is constant under the conditions of these experiments and as long as this is true the conclusions drawn from the study of net resistance apply also to protoplasmic resistance.

#### SUMMARY.

An electrical current passing through a living plant flows partly through the cell wall and partly through the protoplasm. The relative amounts of these two portions of the current can be calculated.

The outcome of such calculations shows that the conclusions drawn from the study of the resistance of the tissue as a whole apply also to the resistance of the protoplasm, and consequently to the permeability of the protoplasm to ions.

<sup>14</sup>A rough calculation shows that this is also true of  $K_{NP}$  and  $K_{OP}$  (corresponding to the  $K_N$  and  $K_O$  mentioned in the former paper<sup>13</sup>).





# STEREOTROPIC REACTIONS OF THE SHOVEL-NOSED RAY, RHINOBATUS PRODUCTUS.

By S. S. MAXWELL.

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## I.

It has been pointed out by Loeb<sup>1</sup> that our orientation in space is determined mainly by three groups of tropistic influences; namely, light, gravitation, and contact. Light and gravitation cause the orientation of organisms through effects upon muscle tonus. When the lines of force strike the animal obliquely, as for example, when light rays fall unequally on the two eyes, the unequal stimulation causes differences of tonus on the two sides, and the symmetrically placed muscle groups acting with unequal strength, bring about forced changes in the direction of locomotion. When the lines of force coincide with the axis of symmetry, or the plane of symmetry of the body, the effects are equal on the two sides and movement can go forward in a straight line. For the contact, or stereotropic, reactions, quantitative relations of this nature have not heretofore been described.

In my studies on the physiology of the labyrinth I have found it necessary to distinguish carefully between those eye and fin movements which result from excitations of end-organs in the ear, and movements which arise from other sources. In this way I have come to make observations on the contact reactions of the shovel-nosed ray, or guitar fish, *Rhinobatus productus*, which will, I believe, throw important light on the nature of stereotropic reactions in general.

*Rhinobatus* is not so broadly expanded as most of the other rays. The pectoral fins, however, have the characteristic fleshy thickened

<sup>1</sup> Loeb, J., Forced movements, tropisms, and animal conduct, Philadelphia and London, 1918.

base. The posterior part of the body and the tail are shark-like in appearance. The eyes are freely movable and can be elevated or retracted in a manner quite similar to the eye movements of the frog. Specimens 3 to 4 feet in length may be taken, but the reactions about to be described are better seen in the smaller animals, 15 to 18 inches long.

When this animal is placed on a shark board and supplied with plenty of aerated sea water through a rubber tube, little or no tying is necessary to keep it in position. Under these conditions a contact stimulus applied to the upper surface of the head or snout excites certain very definite coordinated movements of the fins and eyes, the particular combination of movements depending on the locus and strength of the stimulus.

## II.

If the skin of a *Rhinobatus* is gently stroked with the finger or with a blunt instrument at any point along the midline of the head, for example, between 7 and 8 (Fig. 1), both eyes are retracted, the movements of the two being approximately equal. If a similar stimulus is applied near the outer margin of the upper surface of the head, as at 1 (Fig. 1), the eye on that side is retracted strongly, the other eye is moved very little or not at all. If trials are made at other places, *e.g.*, at 2 or 3, Fig. 1, it is seen that as the point stimulated approaches the midline the amount of movement of the two eyes becomes more and more nearly equal, or in other words, the relative amount of retraction of each eye varies inversely with its distance from the point of application of the stimulus.

It was relatively easy to record these movements graphically. An Engelmann pincette was attached to each eye by a fold of the integument just where the rudimentary lid passes over into the cornea. The pincettes were connected by threads to a pair of light heart levers in such a way that retraction of an eye gave an upward direction to the curve. In the tracing here reproduced, Fig. 2, the upper lever was connected with the left eye and the lower with the right. The writing points were placed as nearly as possible in the same vertical line but in order to make the relations more certain simultaneous ordinates were marked throughout. The small rhythmi-

cal oscillations are respiratory, rather than eye movements. Spontaneous, "voluntary" movements occur occasionally, as between 6 and 7 near the end of the tracing. In this experiment the stimulus

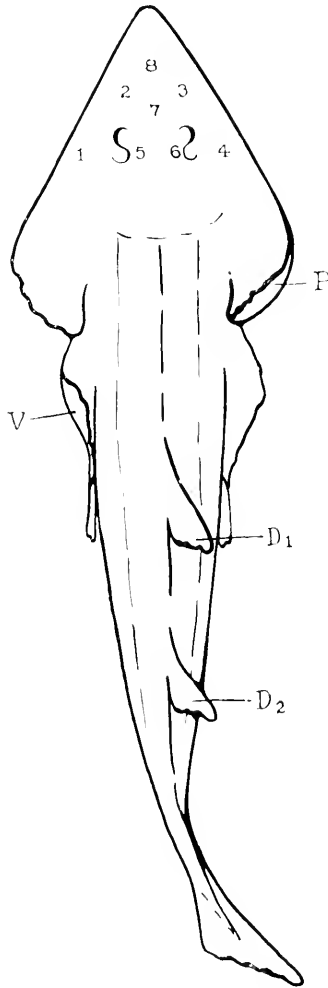


FIG. 1. Diagram of the dorsal view of *Rhinobatus pred. 40*.

employed was a gentle stroke with the finger. These strokes were made as nearly equal as possible, but the method could hardly be expected to give perfectly uniform results. The numbers at the bottom of the tracing show the points stimulated as charted on Fig. 1.

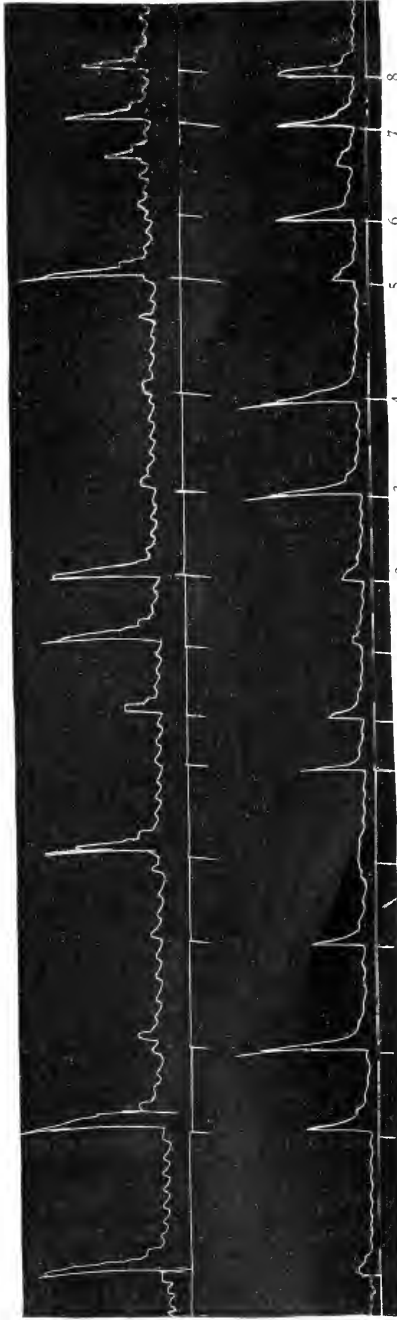


FIG. 2. Upper curve shows movements of left eye; lower curve, right eye. The numbers show the points stimulated, as indicated on Fig. 1.

Certain peculiarities remain to be mentioned. While the responses could be obtained from contact stimuli on all parts of the upper surface of the head, some parts were noticeably more sensitive than others. Also some parts were less likely than others to produce the bilateral response. Thus the strength of stimulus used in securing the tracing reproduced, rarely gave rise to a retraction of both eyes when applied at 5 or 6, very near the inner margin of the eye. Stimuli applied to the lower surface of the snout, even near the lateral margin where the upper surface was very sensitive, were very slightly or not at all effective.

The movements which I have just described are retraction of the bulbs and partial closure of the rudimentary lids, and are not at all to be confused with the conjugate movements which result from excitation of the labyrinth.

The contact stimuli which elicit the eye movements in *Rhinobatus* bring about at the same time a remarkable group of coordinated movements of the fins and tail. An asymmetrically applied stimulus, *e. g.*, at 1 or 2 (Fig. 1), on the left side of the head, causes elevation of the posterolateral margin of the right pectoral, *P*, and of the left pelvic fin, *V*, while both dorsal fins, *D*<sub>1</sub> and *D*<sub>2</sub>, are flexed to the right. A slightly stronger stimulus causes, in addition, a bending of the tail to the right and a slight elevation of the anterolateral margin of the left pectoral fin. If the stimulus is applied to the right side all the relations are, of course, reversed; the left pectoral and right pelvic fins are elevated and the dorsal fins and tail are turned to the left. If the animal was moving forward in the water the effect of the new positions of the fins would be to alter the direction so as to terminate the contact with the stimulating object. If for example the point touched was at 2, Fig. 1, on the left upper surface of the head, the left side of the head and body would be lowered and at the same time the animal would veer off to the right; in other words a definite, negatively stereotropic, reaction would result. The fin and eye movements are as clear and characteristic in their way as are those which result from stimulation of the labyrinth.

In the preceding paragraph I have described the effect of a moderate stimulus. If a more severe stimulus is applied, whether to the midline or to a point asymmetrically situated on the upper

surface of the head, a different reaction results; the margins of all four of the paired fins are elevated strongly. The effect of this would be to check the forward movement in the water and at the same time to steer the fish downward to the bottom. This is plainly also an example of a negatively stereotropic reaction. (It is necessary to bear in mind that a stimulus on the upper midline of the head is symmetrically placed with reference to the median plane of the animal but not with reference to the horizontal plane.)

All of the above reactions occur with great regularity and may be called forth over and over again by appropriate stimuli, but it is important to remember that the character of the response depends not only on the location but also on the nature of the stimulus. A stimulus at a given point may cause a change of direction of locomotion in the horizontal plane with a slight rotation of the body around its longitudinal axis, while a stronger stimulus at the same point may cause a change of direction out of the horizontal plane, that is, a movement toward the bottom.

### III.

The above described reactions occur equally well in animals in which the forebrain has been destroyed. I have made repeatedly transections of the brain as far back as the optic chiasma without affecting them in the least. Complete destruction of the two labyrinths is equally without effect. Since these movements occur in the absence of the forebrain it would be illogical to speak of them as "voluntary," or "purposeful," or "instinctive." On the other hand they illustrate beautifully the tropistic conception of animal behavior since they are very evidently reactions of the organism as a whole in response to asymmetrically applied stimuli. The effect of these stimuli is to bring about sudden changes of tonus in those groups of muscles which in their state of resting equilibrium hold the eyes and fins in a position of symmetry. The change of tonus causes an unsymmetrical action of the corresponding muscle groups on the two sides of the body with the result that the new position induced, terminates the contact with the stimulating object.

At first sight it might appear that these reactions differ in their nature from the other tropisms because in the latter we are concerned

with the influence of forces acting along definite lines, while in the contact reaction the stimulus is applied to a single spot or a limited area of the skin. It is hardly necessary to point out that in heliotropic animals with two eyes the light rays act upon two very limited areas, namely, portions of the retinas, and in the geotropic reactions, gravitation acts upon very limited areas in the internal ear to bring about or maintain orientation. While ordinarily the two eyes or the two ears come into play in the heliotropic and geotropic reactions respectively, experiments show that marked effects are produced by stimuli applied to one eye or one ear alone. Another apparent difference is that contact stimuli may act from moment to moment in different directions. This, however, would be paralleled by the effect of an intermittent, moving light upon a heliotropic organism.

It is of interest to picture the behavior of the organism under the play of two tropistic influences. Instead of a direct response to either, the position or movement which occurs may be the simple resultant of the two, as in the case of barnacle larvæ exposed to two lights from different directions.<sup>2</sup> On the other hand the one stimulus suddenly applied may for the moment inhibit the effect of the other. The free swimming fish, for example, reacts to gravitation by definite compensatory movements and positions of the eyes and fins through which it maintains a definite course and a horizontal position. If now suddenly a foreign body comes in contact with a certain portion of the head, say a point on the left upper surface, a negatively stereotropic movement occurs; the fins are thrown into an unsymmetrical position causing the left side of the head to be lowered and the course to be changed to the right. These changes terminate the contact and the stereotropic reaction ceases. But the sudden swing to the right has excited the ampulla of the right horizontal canal and a compensatory movement to the left, *i.e.*, to the original course, is produced while at the same time the rotation around the longitudinal, body axis has stimulated the otolith organs and the ampullæ of the vertical canals in such way that the horizontal position is again attained. The resulting behavior of the animal would in this way

<sup>2</sup> Loeb, J., and Northrop, J. H., Heliotropic animals as photometers on the basis of the validity of the Bunsen-Roscoe law for heliotropic reactions, *Proc. Nat. Acad. Sc.*, 1917, iii, 539.

give the appearance of volition and purpose, where a more exact analysis shows its purely mechanical nature. In case more than two sets of influences come into play simultaneously the behavior becomes more variable, the analysis becomes more difficult, and the behavior gives the appearance of purpose or caprice, although its correct interpretation would involve no new factors such as "will" or "intelligence," but merely the recognition of a larger number of variables.



THE STEREOTROPISM OF THE DOGFISH (*MUSTELUS CALIFORNICUS*) AND ITS REVERSAL THROUGH CHANGE OF INTENSITY OF THE STIMULUS.\*

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In the preceding paper<sup>1</sup> I have described certain contact reactions of an elasmobranch fish, *Rhinobatus productus*, and I have shown that these reactions are definitely tropistic in their nature. I pointed out that in *Rhinobatus* the response to a particular stimulus depends upon two factors (*a*) the strength and (*b*) the location of the stimulus; that, for example, a weak stimulus applied to the right upper surface of the head causes the fins to assume an asymmetrical position of such character that the body momentarily swerves to the left, and at the same time the right side of the head is depressed; but a strong stimulus applied at the same point brings about such a change in the position of the fins as would arrest the forward movement of the animal and cause it to dive to the bottom. The effect of both these modes of reaction is to terminate the contact with the stimulating object and hence they are both to be regarded as examples of negative stereotropism.

When I attempted to find out whether analogous reactions could be obtained from other elasmobranchs I was at first greatly puzzled by the behavior of the common dogfish. A dogfish tied down on the shark board and supplied with a current of aerated sea water would respond to stroking or scratching stimuli applied to the head or snout with decided movements or changes or position of the fins; but the results were often confusing or contradictory. A contact

\*The expense of this research has been met in part by an appropriation from the Board of Research of the University of California.

<sup>1</sup> Maxwell, S. S., Stereotropic reactions of the shovel-nosed ray (*Rhinobatus productus*), *J. Gen. Physiol.*, 1921-22, iv, 11.

stimulus applied to the right upper surface of the snout would at one moment cause the dorsal fins to turn to the right, while at another moment a stimulation of the same region caused these fins to bend to the left. The paired fins and the tail participated in these responses, and the direction of their movements had a definite relation to the movements of the dorsal fins. It became apparent that these fin movements were always consistent among themselves; they were more than simple reflexes, and showed a coordinated adjustment of the organism as a whole. In general they could be seen to exhibit such an arrangement as would be necessary to turn the animal either in the direction of the stimulating object or away from it. That is to say, the reactions were in each case stereotropic, but the sense of the stereotropism could be positive or negative. It became then a matter of interest to determine, if possible, the conditions of the reversal, and so to control these conditions as to make the responses predictable. This proved to be indeed very simple.

#### METHODS.

In making these experiments on the effects of contact stimuli it would have been desirable to keep the fish in its natural position in the water. This however was impracticable because the mechanical effect of the stroke or push which constitutes the stimulus was sufficient to move the body of the fish under the unstable conditions of water support only. Moreover the stimulus excited movements of locomotion and the observer was unable to keep track of the positions and changes of positions of the different fins. If the aquarium used was large the fish was soon out of reach; if small, new stimuli were offered by collision with the walls. Another disturbing factor, if the animal is floating in the water and free to move, is the fact that each response to a tactile stimulus causes such a change of position as to excite the labyrinth and thus introduce other reflexes. It was necessary, therefore, to use the ordinary method of artificial respiration by means of a current of aerated sea water through a rubber tube in the animal's mouth.

When the dogfish is first placed in the shark board rather violent struggles occur, and tying is usually necessary until the animal be-

comes quiet. After a few minutes of immobility the cords can be gently loosened and removed and the experiment can go on for some time without any need of artificial restraint. This is important because experiments on contact stimuli should not be complicated by possible inhibitions or reenforcements from the presence of the binding cords. It is true that the ventral surface of the body is still in contact with the board, but this is not an unnatural situation since the animal when free often rests for long periods on the bottom of the aquarium. In order better to observe the movements of the paired fins the animal was usually placed above the board on a thick piece of wood no wider than the body, thus allowing the pectorals to project like wings.

The reactions about to be described were obtained by stroking or scratching the outer margin of the head from near the snout to a point just below the eye. It was not necessary that the stroke be carried the whole distance; a short stroke or sometimes a mere touch anywhere within the region mentioned gave the same result. It is not to be inferred that analogous reactions are not elicited by contact stimuli applied to other regions. I have confined this paper to reactions from the parts mentioned for the sake of definiteness of description and interpretation.

#### *Strength of Stimulus and Sense of Reaction.*

For most dogfish a stroke with a finger wet with sea water was sufficient to produce a definite response. As a more severe stimulus I used a scratch with the points of a small pair of forceps. The first of these usually corresponds to the designation "weak" the other "strong" stimulus.

It soon became apparent that fairly constant responses could be obtained if the stimuli were of uniform intensity. In fact under favorable conditions the movements could be repeated over and over with machine-like regularity. The following portion of the record of an experiment is typical (Table I). The pauses between the successive trials were merely the time necessary to set down the results.

*Weak Stimuli.*—Inspection of the results of the above experiment shows that when a weak stimulus is used the dorsal fins and the

tail turn toward the stimulated side. The effect of these as a steering apparatus would be to change the course toward the stimulated side; e.g., turning the dorsal fins or the tail to the left would cause the course to swerve to the left. But in addition to this another effect

TABLE I.

*Mustelus californicus*, 33 Inches Long, May 20, 1921.

Stimulus.		Reaction.				
Kind.	Side.	D1.	D2.	Tail.	Right Pectoral.	Left Pectoral.
Weak (Finger).	Left.	Left.	Left.	Left.	Down/	Up\
	Right.	Right.	Right.	0	Up\	Down/
	Left.	Left.	Left.	Left.	Down/	Up\
	Right.	Right.	Right.	Right.	?	Down/
	Left.	Left.	Left.	Left.	Down/	Up?
	Right.	Right.	Right.	?	Up\	Down/
Strong (Forceps).	Left.	Right.	Right.	Right.	Down/	Up/
	Right.	Left.	Left.	Left.	Up/	Down\
	Left.	Right.	Right.	?	Down\	Up/
	Right.	Left.	Left.	Left.	Up/	Down\
Weak (Finger).	Left.	Left.	Left.	Left.	Down/	Up\
	Right.	Right.	Right.	Left?	Up\	Down\
	Left.	Left.	Left.	0	Down/	Up\
	Right.	Right.	Right.	Right.	Up\	Down/
Strong (Forceps).	Left.	Right.	Right.	Right.	Down\	Up/
	Repeated many times over with like results.					

The first column indicates the strength of stimulus; the second, the side of the head to which it is applied; the third, fourth, and fifth, the direction of movement of the first and second dorsal and the tail fins respectively. The last two columns give the direction of movement of the anterior border of the right and left pectoral fins; and, in these two columns, / at the end of the word indicates that the posterior end of the fin was higher than the anterior; \, that the posterior margin was lower than the anterior.

would result. When a dorsal fin turns to the left it assumes an oblique position; that is, it is its posterior border which goes to the left most strongly. Its resistance as the animal moves forward in the water would have a screw effect, tending to rotate the body around its longitudinal axis so that the ventral side would be turned in the di-

rection of the stimulating object. This rotation effect would be increased by the new position of the pectoral fins. The pectoral on the stimulated side is elevated but its posterior margin is raised less than its anterior or is even depressed; the pectoral of the other side makes a movement which is just the converse. These fins would then also have a screw effect tending to the same direction of rotation as the dorsals, namely, ventral side toward the stimulating object. The reaction is clearly tropic and in the positive sense.

It will be seen that the total effect of a weak stimulus is to turn the ventral side of the animal, as well as to swerve the course, in the direction of the stimulating object. This accords well with what one sees on watching the dogfish swimming about in a small aquarium. They are often seen going round and round, keeping near the walls, with the body tilted to one side so that the mouth and belly are turned somewhat toward the wall. This is just the position which would be produced by the above reactions, if, on making the turn at a corner, the edge of the snout came slightly in contact with the wall. Sometimes I have been able to see such contacts actually occurring, but the asymmetrical position was often assumed when the wall was not touched. In this case it might be that the increased pressure or resistance of the water when the fish was moving near the wall could act as a stimulus. Indeed I found that a spurt of water from a pipette could be used instead of a finger stroke as a weak stimulus.

Since in the dogfish the mouth is far back on the ventral surface of the head it is not unreasonable to suppose that the positive stereotropic reaction assists in the capture of food; the response to a contact stimulus would tend at once to bring the mouth into position to seize the stimulating object.

*Strong Stimuli.*—The experiments described above show that the reaction to a strong stimulus is almost exactly the reverse of the reaction to a weak stimulus. The dorsal fins and the tail are flexed to the side away from the contact. The pectoral fin on the stimulated side is elevated, its posterior margin still more than its anterior, the pectoral on the opposite side is depressed, the posterior margin more than the anterior. The whole arrangement of the fins is that of a screw whose effect in the water would be to rotate the body around its longitudinal axis in such a way as to turn the back to the stimulat-

ing object. At the same time the dorsal fins and the tail would act as a steering apparatus to alter the course to a direction away from the source of stimulation. This then is also a definite tropic reaction and in the negative sense.

Attention should perhaps be called to the fact that while the positive reaction is on the whole opposite in character to the negative it is not precisely so. A weak stimulus on the right side of the snout or a strong stimulus on the left side would each tend to turn the ventral side to the right and cause the course to veer to the right. But the mechanism is not quite the same so far as the pectorals are concerned. In the positive reaction the pectorals act feebly in comparison to the unpaired fins; in the negative reaction their movement is relatively more vigorous. In both the positive and the negative reaction the pectoral of the stimulated side is elevated; but in the one case its posterior margin is elevated less than the anterior and in the other case more. It will not do then to say that the negative reaction differs from the positive merely in the fact that the excitation is shunted from one side of the central nervous system to the other. It is certainly not so simply diagrammatic as that.

#### *The Decerebrate Animal.*

My experiments on *Rhinobatus* led me to expect that the destruction of the forebrain would have no effect on the character of the stereotropic reactions of the dogfish. In a number of instances I made transections of the brain, usually near the anterior margin of the cerebellum, with no noticeable alteration in the responses to contact stimuli. The following record of an experiment will serve as an example:

July 4, 1921.—*Mustelus californicus*, 29 inches long.

9:20 a.m. Brain exposed and cut across at anterior margin of cerebellum. Animal returned to tank, lies inert; does not right itself.

10:30 a.m. Animal swimming about normally. Taken out and placed on board. Contact reactions tested. (Table II.)

#### *Possible Sources of Error.*

Certain possible sources of error were considered and should be mentioned.

1. *Reflexes from the Labyrinth*.—I have already spoken of the necessity of avoiding any movement of the head at the moment of the experiment. It would be quite possible, if labyrinth effects were not taken into account, that the mechanical effect of the stroke used as the stimulus would turn the head enough to excite a reflex from the internal ear. I have often in the course of an experiment tried to see how much and how fast the head must be turned in order to provoke a labyrinthine response, and the amount has always been much greater than could be caused by the strongest contact stimulus employed. But in order to avoid all possibility of error from this source I have made experiments on a number of dogfish in which both labyrinths

TABLE II.

Stimulus.		Reaction.				
Kind.	Side.	D1.	D2.	Tail.	Right Pectoral.	Left Pectoral.
Forceps scratch.	Right.	Left.	Left.	Left.	Up.	Down.
	Left.	Right.	Right.	Right.	Down.	Up.
	Right.	Left.	Left.	Left.	Up.	Down.
	Left.	Right.	Right.	Right.	Down.	Up.
Finger stroke.	Right.	Right.	Right.	0	Up.	Down.
	Left.	Left.	Left.	Left.	Down.	Up.
	Right.	Right.	Right.	Right.	Up.	
	Left.	Left.	Left.	Left.	Down.	Up.
Continues to respond like a normal animal.						

had been previously destroyed and have found the stereotropic responses in no way altered.

2. *Tension of Neck and Trunk Muscles*.—The observation of Lyon<sup>2</sup> that eye movements can be elicited by bending the body of the dogfish, even after total destruction of the ears, can be easily repeated. Fin movements can also be obtained in the same way. It was conceivable, then, that the responses or some of them might have been due to pressure on the side of the head inducing reflexes by changes of tension in the joints of the neck region. It was easy to test this also

<sup>2</sup> Lyon, E. P., Compensatory motions in fishes, *Am. J. Physiol.*, 1900-01, iv, 77.

and I found that the amount of bending necessary to produce any reflex movement in this way was vastly more than could be caused by the contact stimuli.

3. *Retinal Stimuli*.—I have already mentioned the fact that dogfish swimming about in the concrete tank often keep close to the wall, with the dorsal fins slightly flexed and the body tilted so that the belly is turned slightly outward toward the wall. It was possible that this position was induced by the moving image of the wall upon the retina. In making the strokes used as contact stimuli I naturally passed my hand close to the eye of the stimulated side. It was possible that the flexion of the dorsal fins might be due in reality to the image (or the shadow) of the hand upon the retina. I found in fact that when the fish was placed on the board parallel to the window, so that the left eye was toward the light, passing the hand between the window and the animal's left eye often caused a definite flexing of the first dorsal fin to the left, but I could never by this means get a movement of the other dorsal or of the paired fins. In order, however, to test this matter farther I made many experiments in which the eyes were covered with thick pads of wet absorbent cotton and found no apparent change in the responses to contact stimuli.

#### CONCLUSIONS.

In the majority of instances the regularity of the responses to the finger strokes and to the forceps scratches is no more remarkable than the definiteness of the change from the one kind of response to the other. The two kinds of stimuli mentioned differed sufficiently to give reactions of opposite sign. An occasional animal, however, reacted very feebly or not at all to the finger stroke, and gave "positive" reactions to fairly strong forceps scratches, in fact in a few instances no negative reaction was obtained. Other specimens gave positive reactions to moderately strong forceps scratches (stimuli which in the great majority of specimens would cause a lively negative reaction), but gave the negative reaction when still more force was applied. On the other hand a few gave only the negative reaction to any effective stimulus.

It was noticeable, too, that what constituted a "weak" or a "strong" stimulus depended upon the physiological state of the individ-



ual animal. Specimens were usually less sensitive and less responsive immediately after the struggles connected with capture and immobilization were over than they were ten or fifteen minutes later. In fact an occasional animal remained unresponsive until roused to excitability by an unusually hard scratch or a pinch or twist of the tail, when it suddenly began to react in the regular way. These instances forcibly reminded one of the awakening from a nap. Immediately after the "awakening" all the responses, even to strong stimuli, were likely to be positive, although occasionally just the reverse was the case. Then after a few strokes the reactions would become normal, that is, positive to weak and negative to strong stimuli.

Naturally one raises the question: How is the reversal brought about? The phenomena described in this paper seem to present a close analogy to the observation of Sherrington<sup>3</sup> that in a spinal dog the reaction to a stimulus applied to the plantar surface of the hind foot differs in a way dependent on the nature of the stimulus; a firm gentle pressure causes extension, a sharp prick causes flexion. Sherrington apparently assumes the existence of one kind of nerve endings, nociceptors, which are excited by harmful stimuli, and which give rise to flexion, and another kind which respond to bland stimuli by extension. The analogy in the case of the dogfish is quite marked, except that the stimulus which is "bland" in one animal or in one state of the animal, is "nocuous" in another animal or in another state of the same animal.

There appears to me to be a yet closer analogy between these reactions and the reversibility of the heliotropic reactions of certain organisms; namely, those which are positive to weak and negative, to strong light. All the phenomena seem to me to indicate that the reversal of the stereotropic reactions of the dogfish is a central process. It has been objected to the idea that the heliotropic reversals are brought about in the nervous system that such reversals occur in unicellular organisms where no separate nervous system exists; but it has been pointed out by Loeb<sup>4</sup> that even in unicellular organisms

<sup>3</sup> Sherrington, Charles S., *The integrative action of the nervous system*, New Haven, 1906.

<sup>4</sup> Loeb J., *Forced movements, tropisms, and animal conduct*, Philadelphia and London, 1918.

structures could exist which would have the effect of synapses. It must be admitted, however, that notwithstanding the closeness of the analogy, the reversal of the heliotropic and of the stereotropic reactions may be due to totally different mechanisms.

#### SUMMARY.

1. The dogfish responds to certain contact stimuli by definite stereotropic reactions. These reactions can be positive or negative.

2. The sense of the stereotropic response depends on the strength of the stimulus; a "weak" stimulus, produces a positive and a "strong" stimulus, a negative response.

3. The strength of stimulus necessary to cause a reversal of the reaction depends in part on the physiological state of the animal.

4. The stereotropic reactions occur equally well in the absence of the forebrain.

# CHEMICAL STIMULATION OF THE NERVE CORD OF LUMBRICUS TERRESTRIS.

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Nerve cells of different functions cannot be differentiated histologically by staining methods. In order to prove chemical differences it is necessary to affect the function of a given type of neuron by the action of a chemical agent. Thus Baglioni<sup>1</sup> has demonstrated a chemical difference between sensory and motor cells in the frog and the squid, by showing that phenol is an excitant for the motor ganglia alone while strychnine stimulates the sensory ganglia only of these forms. Maxwell<sup>2</sup> has shown that the nerve cells of the mammalian cortex are stimulated by one class of substances which includes creatine and strychnine, but that such substances do not act on medullated nerve fibers. Only certain salts, such as the calcium precipitants and barium compounds, act upon medullated fibers. On the other hand these salts do not stimulate the gray matter of the brain. Tetraethyl-ammonium chloride is an exception in that it acts upon structures of both types.<sup>3</sup>

It is possible that chemical differences exist between nervous systems of various forms.<sup>4</sup> For example, coelenterates give no spasm response to strychnine, but echinoderms do, provided the concentration of alkaloid is sufficiently high, while cephalopods are as sensitive to strychnine as vertebrates are. On these grounds Parker<sup>5</sup> has suggested that strychnine might be used as a test for the presence of synapses, since sensitivity to strychnine on the part of different

<sup>1</sup> Baglioni, S., *Z. allg. Physiol.*, 1905, v, 43.

<sup>2</sup> Maxwell, S. S., *J. Biol. Chem.*, 1906, ii, 183.

<sup>3</sup> Maxwell, S. S., *Am. J. Physiol.*, 1918-19, xlvii, 283. Loeb, J., and Ewald, W. F., *J. Biol. Chem.*, 1916, xxv, 377.

<sup>4</sup> Moore, A. R., *Proc. Nat. Acad. Sc.*, 1917, iii, 598.

<sup>5</sup> Parker, G. H., *The elementary nervous system*, Philadelphia, 1919, 208.

phyla develops hand in hand with increasing complexity of the nervous system.

By such a method it seems possible to discover similarities and relationships in the chemical constitution of neurons which would otherwise remain undetected. With this purpose in view experiments have been carried out on chemical stimulation of the nerve cord of the earthworm, *Lumbricus terrestris*.

In an experiment the animal was decapitated, pinned down by the anterior end and the anterior portion of the nerve cord laid bare for a distance of about 2 cm. A piece of cord about 1 cm. in length was then separated from the underlying tissue. The substance to be tested was applied to the loosened part. Stimulation was shown by spasmodic squirming of the posterior segments of the worm. Control experiments were made by applying the stimulating substance to a part of the body wall after removal of the nerve cord from that section of the worm. No reaction of the posterior segments resulted.

It is of course impossible to separate the nerve cells from their processes in this form. Therefore excitants of the first class,<sup>2</sup> *i.e.*, calcium precipitants and barium salts, were effective in causing stimulation.  $\text{BaCl}_2$  and  $\text{KCl}$  in concentrations isosmotic with the worm's blood caused strong responses immediately. Responses due to the action of  $\text{Na}_2\text{SO}_4$  and  $\text{Na}_3$  citrate were weaker but unmistakable. Tetra-ethyl-ammonium chloride in  $\text{M}/64$  concentration made up in Ringer solution acted as a powerful excitant.

Of the substances belonging to the second group, camphor in one-fifth saturated solution, strychnine in saturated solution, atropine sulfate in  $\text{M}/8$  concentration and picrotoxin crystals all caused strong reactions. But creatine, caffeine, and nicotine phenol had no stimulating action. Creatine, caffeine, and phenol were applied to the nerve cord in the form of crystals and solutions; nicotine in concentrations of 0.004 per cent and 0.4 per cent was made up in isosmotic Ringer solution.

When chemical excitation did take place the response was almost immediate—within a minute of the time of application. This is a noteworthy fact since in chemical stimulation of the mammalian cortex the latent period is 10 or 15 minutes and the latent period for

the action of nicotine in squid is 6 or 7 minutes at 24°C., the concentration of nicotine being 1,500,000.

In conclusion the facts presented suggest, (*a*) that the nerve processes of *Lumbricus terrestris* are similar in chemical constitution to the axis cylinders of the medullated nerve fibers of mammals; (*b*) that the neurons of the earthworm are more limited in their possibilities of chemical stimulation; *i.e.*, they are simpler in chemical constitution, than the neurons of cephalopods and of mammals.



# THE FORMATION OF THE ASTER IN ARTIFICIAL PARTHENOGENESIS.\*

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In normally fertilized eggs the development of the aster is attributed to a substance carried into the egg by the spermatozoon. The aster first makes its appearance in the form of diminutive radiations surrounding the neck-piece of the spermatozoon within a few minutes after it has entered the egg. The writer<sup>1</sup> has shown that the formation of the radiations is accompanied by a jellying of the cytoplasm of the egg. The jellying process extends more and more as the aster increases in size and the entire egg becomes involved when the center of the aster comes to occupy the center of the egg.

The formation of the aster is accompanied by an increase in size of a hyaline area in its center. This is Wilson's hyaloplasm-sphere<sup>2</sup> also called centrosphere and astrosphere by other investigators. The microdissection method has demonstrated that this sphere area is liquid in contrast to the surrounding jellied cytoplasm. The pioneer observers of mitotic division, such as Auerbach, Hertwig, Bütschli and Fol, described the accumulation of a hyaline plasma at the astral centers and suggested that the astral radiations are a result of protoplasmic currents. Later investigators, such as Morgan, Wilson and Conklin, considered this view as the most probable one.

\*The experiments, upon which this paper is based, were conducted in the Research Division of Eli Lilly and Company, at the Marine Biological Laboratory, Woods Hole. The experiments constitute a part of a joint research project in which Dr. G. H. A. Clowes and the writer are engaged.

<sup>1</sup>Chambers, R. Microdissection studies. II. The cell aster: A reversible gelation phenomenon, *J. Exp. Zool.*, 1917, xxiii, 483.

<sup>2</sup>Wilson, E. B., Experimental studies in cytology. I. A cytological study of artificial parthenogenesis in sea-urchin eggs, *Arch. Entwcklungsmechn.*, 1901, xii, 529.

The movement of the egg nucleus is possibly also a case in point. As long as the egg nucleus is beyond the confines of the aster it is stationary. As soon, however, as the extending aster reaches it, the nucleus begins travelling toward the sphere in which it finally lies close beside the sperm nucleus. The existence of a centripetal current may be inferred also from the following experiment. In an egg one may occasionally see one or more oil-like droplets 2 to 4 microns in diameter. If one of these droplets be pushed by the needle from the liquid cytoplasm into the periphery of the aster the droplet will move along the rays toward the center.

In view of the above observations it is highly probable that the liquid which accumulates in the center of the aster streams into it from all sides during the jellying of the cytoplasm. It is this streaming which probably occasions the innumerable radiations characteristic of the aster. After the aster has attained its full size the radiations begin to fade from view as the jelly state reverts to a more fluid one. The liquid of the central sphere does not mix with the fluid cytoplasm but separates into two areas, one at each pole of the mitotic figure of the dividing nucleus. Astral radiations now appear about the two areas as the egg cytoplasm jellies again with the formation of two jellied masses instead of one, as heretofore. These grow at the expense of the fluid cytoplasm until all of the cytoplasm of the egg is taken up into two bodies, the two blastomeres of the segmenting egg.

During the rapidly succeeding cleavages of the egg there is always a cap of liquid on the nucleus of each blastomere. With each mitosis this liquid flows around the nucleus to accumulate in two areas at the poles of the mitotic figure. These areas are periodically augmented during the formation of an aster and the ensuing jellying process.

There is every evidence<sup>3</sup> that the mechanism of cell division depends upon a readiness of the cytoplasm to pass from a liquid to a

<sup>3</sup> Heilbrunn, L. V., Studies in artificial parthenogenesis. II. Physical changes in the egg of *Arbacia*, *Biol. Bull.*, 1915, xxix, 149; An experimental study of cell division. I. The physical changes which determine the appearance of the spindle in sea-urchin eggs. *J. Exp. Zool.*, 1920, xxx, 211; Chambers, R., Changes in protoplasmic consistency and their relation to cell division, *J. Gen. Physiol.*, 1919, ii, 49.



jellied state and *vice versa*. The protoplasm must have its phase relations in a delicately balanced state in order that this may occur. In the egg we have seen that the reversal to a jellied state is probably accompanied by a separating out of a liquid. Something in this liquid may possibly control, in periodic rhythms, the physical state of the protoplasm surrounding it. We may assume that as long as there is a quantity of this substance localized in the egg it can induce aster formation. The idea suggests itself that one purpose of the spermatozoon is to accumulate this substance. In the mature unfertilized egg there is no localized area from which the jellying process may spread. The entrance of a sperm furnishes a focus as it were. Around this focus an aster develops with a steady accumulation of the liquid in its center. This liquid area surrounds the nucleus and puts the egg in a condition similar to that of a blastomere. The process of cleavage then becomes the same in both.

An interpretation dissonant with previous ones concerning the mode of aster formation in artificially parthenogenetic eggs has been recently put forward by Herlant.<sup>4</sup> Wilson<sup>2</sup> in *Toxopneustes*, had long ago shown that eggs treated insufficiently with a parthenogenetic agent may form monasters which disappear and reappear in several successive rhythms. Hindle<sup>5</sup> found this to be true also for the sea-urchin egg, if treated with butyric acid alone. A sufficient treatment, however, of a parthenogenetic agent results in the disappearance of the monaster followed by the appearance of an amphiaser. This results in cleavage of the egg. In the sea-urchin egg, the butyric acid treatment has to be followed by a bath of hypertonic sea water in order that this may occur. The hypertonic treatment often results in the formation of several cytasters in the egg. The cytasters produced by the hypertonic treatment Herlant claimed to be due to dehydrative effects producing spots within the egg cytoplasm about which the asters appear. Herlant assumed that one of these cytas-

<sup>4</sup> Herlant, M., Comment agit la solution hypertonique dans la parthénogenèse expérimentale (méthod de Loeb). I. Origine et signification des asters accessoires. *Arch. Zool. exp. et gén.*, 1918, lvii, 511; II. Le mecanisme de la segmentation. *Arch. Zool. exp. et gén.*, 1919, lviii, 291.

<sup>5</sup> Hindle, E., A cytological study of artificial parthenogenesis in *Strongylocentrotus purpuratus*, *Arch. Entwicklungsmechn.*, 1910-11, xxxi, 145.

ters connects in some way with the monaster, thus forming the amphia-ster which initiates segmentation. The weakness in this interpretation is the lack of conclusive evidence for the union of the originally independent asters. Neither Wilson nor Hindle ever observed such a phenomenon. All my observations also indicate that the amphia-ster in parthenogenetic eggs arises from a previous single aster just as it does in normally fertilized eggs.

My studies were mainly confined to the egg of the sand-dollar. In its behavior to parthenogenetic agents<sup>6</sup> the egg is almost identical with that of the sea-urchin which Herlant studied. The absence of pigment and the highly translucent nature of its protoplasm makes the sand-dollar egg an ideal object for observational study.

The mature eggs, normally shed by the female, are placed in butyric acid (2 cc. 1/10 N in 50 cc. of sea water) for 35 seconds. During this treatment the eggs distinctly round up. They are then returned to sea water where, within a few minutes, the fertilization membrane lifts off. After 20 minutes the eggs are placed in hypertonic sea water (5 cc. 2.5 M NaCl in 50 cc. sea water). The eggs shrink slightly in this solution. After 20 minutes the eggs are transferred to a large quantity of normal sea water and the sea water is changed several times to free the eggs from any further action of the hypertonic solution.

Up to this time no change whatever is to be seen in the cytoplasm or in the nucleus. While in the hypertonic solution the cytoplasm appears more granular and opaque than that of an untreated mature egg. However, on the return of the treated eggs to sea water the cytoplasm reverts to its former appearance and to the eye the eggs differ in no respect whatever from unfertilized eggs except for the presence of a fertilization membrane.

It is not until the treated eggs have stood in sea water for several minutes that any cytoplasmic change is to be observed. The first sign of a change consists in the appearance of faintly defined vacuoles about the center of the egg. Within a few minutes they coalesce to form a central clear area of about one-tenth the diameter of the

<sup>6</sup> Just, E. E., The fertilization reaction in *Echinarachnius parma*. III. The nature of the activation of the egg by butyric acid. *Biol. Bull.*, 1919, xxxvi, 39.

egg. The egg nucleus lies close to or within this area. Gradually rays begin to appear in the jellying cytoplasm about the area. These rays become more numerous and more pronounced until the entire egg is occupied by a large monaster which corresponds exactly with the fully developed sperm aster of a normally inseminated egg. From now on the process is entirely analogous to that of a sperm fertilized egg. During the development of the aster the hyaline central area increases in size and the microdissection needle shows it to be a liquid area characteristic of that of the sperm aster. When the monaster disappears the liquid central area flows around the nucleus now undergoing mitosis and accumulates at the two poles of the nucleus into two polar areas. A jellying process now sets in with these two areas as centers and results in the amphiaser preparatory to the first cleavage of the egg.

In the mode of aster formation the only difference between the sperm fertilized and the parthenogenetic egg consists in the manner in which a liquid separates out of the jellying protoplasm in connection with the formation of the preliminary single aster. In the fertilized egg radiations appear immediately about the sperm-head and the accumulation of the liquid substance is from the beginning through the agency of the ray-like channels of the growing aster. In the parthenogenetic egg several vacuoles first appear in the cytoplasm. These vacuoles collect in the center of the egg after which an aster appears.

The frequent irregularities which obtain in parthenogenetic eggs are apparently due to an incomplete fusing of the vacuoles and to a lack of polarity in the preliminary stages of the aster formation. In undertreatment, or when butyric acid alone is used, a monaster develops as usual. Upon the disappearance of the monaster, the persisting liquid centrosphere, instead of flowing to the two polar regions of the nucleus, remains a single body. With the return of the jellying period a single aster again forms and more fluid accumulates in the centrosphere which increases in size. This process repeats itself several times and segmentation of the egg never occurs.

Eggs treated with butyric followed by a prolonged treatment of the hypertonic solution become abnormal. In cases of this kind the eggs, when returned to sea water from the hypertonic solution,

exhibit vacuoles which, instead of being collected in the center of the egg, are scattered throughout the cytoplasm. Radiations appear about these vacuoles with the result that the egg becomes filled with many small asters. The longer the eggs have been left in the hypertonic solution the more numerous will be the asters, and most if not all of these asters develop independently of one another. Irregularities may occur, even when the vacuoles collect in the center of the egg. In such cases an apparently normal single aster first results. Upon its disappearance, the central liquid area, instead of flowing away from the center into two polar bodies, produces three or four irregular lobes. About each of these lobes radiations appear in the egg cytoplasm producing a multipolar aster. In one instance one such lobe separated itself from the main body and a complete aster formed about it while a multipolar aster formed about the rest of the hyaline area. When the periphery of a multipolar aster reaches the surface of the egg cleavage furrows form between each lobe of the aster so that such eggs may segment simultaneously into three or four or more blastomeres. Asters which form independently of the central area never seem to be large enough to bring about segmentation of the egg into considerable masses. When such asters lie close to the periphery of the egg, furrows often grow in from the surface of the egg enclosing the asters. In this way a superficial type of segmentation results with the pinching off of small masses of the egg. The development of cytasters resulting in a spurious segmentation has already been described by Wilson.<sup>2</sup>

The first aster appears at about the same time after the acid treatment, irrespective of whether the eggs have been subsequently treated with the hypertonic solution or not. However, with subsequent hypertonic treatment, the reappearance of the radiations following the fading away of the first aster occurs about more than one center. This results in segmentation of the egg. The reaction, therefore, which is peculiar to hypertonic treatment shows up only *after* the disappearance of the first aster. At that time the persisting central liquid area of the aster, instead of remaining as a single centralized mass, separates into two or more bodies with the result that the following reappearance of rays in the cytoplasm occurs as radiations about these bodies. This produces multiple asters. If there be

only two focal points the liquid collects into two bodies, a typical amphiaster then develops, and the egg cleaves into two normal blastomeres.

Aster formation not only consists in a jellying process but also in the separating out of a liquid. The optically visible phenomenon peculiar to the parthenogenetic egg consists in the manner in which this liquid begins to separate out of the egg cytoplasm preparatory to the formation of the preliminary single aster. In the sperm fertilized egg both processes are rapid and occur together, radiations appear immediately about the sperm-head, and the accumulation of the liquid substance is from the very start through the agency of the ray-like channels of the growing aster. In the parthenogenetic egg the jellying process is apparently very slow, and the separating out of a liquid takes place before the cytoplasm is stiff enough to exhibit channels through which the liquid flows to the center. The liquid first collects into several vacuoles and an optimum treatment is necessary to cause these vacuoles to fuse into one body with the subsequent formation of a single aster. Overtreatment causes the appearance of many vacuoles scattered throughout the egg resulting in multiple asters. Undertreatment may result in the formation of a single aster which, however, periodically disappears and reappears as a single aster.

The parthenogenetic treatment, in order to be successful, must not only bring about the separating out of a liquid from the egg cytoplasm, but must also induce polarity within the resulting hyaline area in order to enable it to form two centers about which an amphiaster may develop.



# STUDIES ON THE ORGANIZATION OF THE STARFISH EGG.\*

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The following is a preliminary record of operative work on the starfish egg which throws some light on the nature of the fertilization membrane, the interaction between nucleus and cytoplasm, and the relation of the cortex to the interior of the egg.

By means of the microdissection needle it has been possible to show that a morphologically definite membrane closely invests the unfertilized egg, and that it is this membrane which lifts off upon fertilization as the so called fertilization membrane. The description of two methods will suffice to demonstrate this. By carefully pressing an unfertilized mature egg between the surface of a cover-slip and the side of a slender glass needle the egg may be cut in two without tearing the investing membrane. This membrane now becomes apparent, bridging the gap between the two egg fragments and holding them together. Upon the addition of sperm this membrane lifts off as the fertilization membrane, in such a way that the two egg fragments come to lie within a single cavity.

The unfertilized egg can also be slipped entirely out of its investing membrane. Such an egg will undergo normal fertilization and cleave into blastomeres having no investing membrane whatever.

These two experiments definitely show that the normal unfertilized starfish egg is already surrounded by a membrane which, upon fertilization, becomes the fertilization membrane.

The difference in behavior towards sperm of an egg, which has been denuded not only of its jelly but also of its membrane, and one which has not is very striking. In an egg enclosed in its membrane

\*The experiments reported in this paper constitute a part of the joint investigation of the mechanism of fertilization in which Dr. G. H. A. Clowes and the writer are engaged.

the spermatozoa quickly crowd about the egg as they are trapped in the jelly surrounding the membrane. In a membraneless egg no crowding of spermatozoa is noticeable and heavy insemination is necessary to bring about fertilization. With such eggs, when a cloud of sperm has been blown upon them, one may frequently observe a spermatozoon swim toward an egg, wander over its surface and then swim away. On the other hand the empty membrane with its investing jelly immediately becomes covered with a halo of active spermatozoa.

The nucleus of the egg cell is a liquid drop surrounded by a morphologically definite membrane. The nucleus may be moved about within the egg with the needle, and can be considerably deformed by pressure. On removal of the needle the nucleus quickly resumes its spherical shape. Tearing the nucleus slightly causes the nucleus to shrink and the nucleolus to disappear; this is followed by a remarkable spread of a disintegrative process which involves the cytoplasm surrounding the nuclear area. In the immature egg, where the nucleus is large, the disintegrative process may extend throughout the entire egg. In the mature egg with a relatively small nucleus the destruction is restricted to a limited area.

The disappearance of the nucleus or germinal vesicle during maturation has been described by several investigators. The nuclear membrane breaks down spontaneously and the nuclear sap spreads slowly throughout the cytoplasm. So long as the nuclear area, aside from the definitive egg nucleus, has not yet mixed with the cytoplasm, I find that a puncture of the area starts up the disintegrative process. When the nuclear sap has entirely mixed with the cytoplasm, any part of the egg, with the exception of the minute egg nucleus, may be torn with impunity. The mere presence of the glass needle in the nuclear sap is not sufficient to start up the disintegrative process. This process occurs only when the nuclear sap is agitated by the needle while the sap is in direct contact with the cytoplasm.

Wilson<sup>1</sup> found in the Nemertine egg that any non-nucleated fragment, prior to the dissolution of the germinal vesicle, is non-fertilizable whereas, any fragment from a mature egg is capable of being fertilized and undergoing cleavage. This I have found to be true also for the

<sup>1</sup> Wilson, E. B., Experiments on cleavage and localization in the Nemertine egg, *Arch. Entwicklungsmechn.*, 1903, xvi, 411.



starfish egg. It is also of interest to note that the fertilizability of the egg fragments is directly connected with the extent of the mixing of the nuclear sap with the cytoplasm in the maturing egg. A non-nucleated fragment, taken from an egg in the early stages of the dissolution of the germinal vesicle, will admit sperm which will undergo several nuclear divisions with, at most, an abortive attempt on the part of the fragment to cleave. When the sap of the germinal vesicle has completely mixed with the cytoplasm, any fragment larger than a certain size limit is capable of being fertilized and undergoing cleavage.

It is well known that immature eggs can be kept in sea water at room temperature for 24 hours or more without disintegrating and that unfertilized mature eggs go to pieces under the same conditions within a much shorter time.<sup>2</sup> The writer has found that nucleated fragments of the two kinds of eggs behave similarly, while non-nucleated fragments act quite differently indicating that the substance which prevents the disintegration is distributed differently in the two eggs. Non-nucleated fragments of immature eggs last for about 4 hours only. Similar fragments of mature eggs last from 8 to 10 hours, or about as long as the mature, nucleated fragments. The substance which prevents the destruction of the egg is apparently in the nuclear sap which, in the immature egg, is confined within the large nucleus or germinal vesicle, while in the mature egg this sap has escaped from the nucleus and spread throughout the entire egg.

The following experiments indicate that the part of the starfish egg which is capable of development is chiefly confined to the cortex of the egg. It was long ago shown by Driesch,<sup>3</sup> Loeb<sup>4</sup> and others that starfish and sea-urchin eggs are highly fluid in that fragments quickly round up into spheres. That the cortex of the mature unfertilized eggs is firmer in consistency than their interior has been

<sup>2</sup> Loeb, J., and Lewis, W. H., On the prolongation of the life of the unfertilized eggs of the sea-urchins by potassium cyanide, *Am. J. Physiol.*, 1902, vi, 305. Loeb, J., Maturation, natural death and the prolongation of the life of the unfertilized starfish eggs (*Asterias forbesii*) and their significance for the theory of fertilization, *Biol. Bull.*, 1902, iii, 295.

<sup>3</sup> Driesch, H., Entwicklungsmechanische Studien. Der Werth der beiden ersten Furchungszellen der Echinodermmentwicklung, *Z. wiss. Zool.*, 1891, liii, 60.

<sup>4</sup> Loeb, J., Ueber die Grenzen der Theilbarkeit der Eisubstanz, *Arch. Physiol.*, 1895, lix, 379.

described by the writer.<sup>5</sup> If the surface of the mature starfish egg be torn with a needle, and the egg then caught at the opposite side and pulled to the edge of the hanging drop, the compression on the egg produced by the shallow water at the edge of the drop will cause the fluid interior to ooze out through the tear, forming a perfect sphere. One may so manipulate the process as to cause the egg nucleus either to remain behind in the cortex (the cortical remnant) or to pass into the extruded sphere.

The cortical remnant is relatively solid and remains more or less enclosed within the egg membrane and its jelly. If left long enough it will eventually round up so as to present the appearance of a diminutive egg surrounded by a collapsed and wrinkled egg membrane.

The material which has escaped from the egg into the sea water is fluid and tends immediately to round up. On tearing with a needle its surface behaves like that of a highly viscous oil drop. These spheres adhere tenaciously to glass and, in the effort to remove them by blowing a current of water against them, they sometimes leave a torn off piece behind. The cortical remnant is readily fertilizable and undergoes normal segmentation. On the other hand, the material which has escaped from the interior of the egg whether nucleated or not, is non-fertilizable. It remains inert until it finally undergoes disintegration. As long as it possesses an intact surface it appears exactly like an egg fragment and will undergo disintegrative changes similar to those of entire eggs, on being torn with the needle. If even a small part of the original cortex is allowed to remain continuous with the sphere it is fertilizable and the more cortical material present the more will the sphere approach normal cleavage.

It is significant that the fluid spheres which escape from the interior of the mature unfertilized egg, whether nucleated or not, withstand disintegration for a much longer period than do fragments, containing cortical material, which have been produced simply by cutting an egg into two or more pieces.

It follows from these facts that the part of the starfish egg chiefly concerned in development lies in its periphery. The interior when separated from the cortex is incapable of developing. On the other hand, an egg containing cortical material alone is able to carry on its usual life activities.

<sup>5</sup> Chambers, R., Microdissection studies. I. The visible structure of cell protoplasm and death changes, *Am. J. Physiol.*, 1917, xliii, 1.

# THE SELECTIVE ABSORPTION OF POTASSIUM BY ANIMAL CELLS.

## I. CONDITIONS CONTROLLING ABSORPTION AND RETENTION OF POTASSIUM.

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The accumulation of potassium with little or no sodium in the cell living in a medium richer in sodium than in potassium is so widespread a phenomenon as to be considered a general attribute of living cells. In the few exceptions on record the distribution of sodium between cells and tissue fluids has not been satisfactorily investigated. The mechanism governing potassium selection has not been explained. Loeb (1) as early as 1906, stated, "we may take it for granted that, at least, potassium forms a non-dissociable constituent of the protoplasm of a number of tissues of animals and plants." The view that much of the potassium of the cell is not dissociated seems generally accepted. Robertson (2), for example, uses the selective action of cells for potassium as one of the proofs of the dissociation of protein salts so as to hold inorganic constituents as a part of protein ions without the formation of free inorganic ions. Such a theory explains very well the retention of accumulations of potassium in the cells but it gives no clew as to why potassium is "selected" instead of sodium nor, in the face of the apparent impermeability of normal cells to electrolytes, does it explain how potassium makes its way into growing cells. Our investigations bear on such aspects of the problem.

It seemed of first importance to find some of the limiting conditions for the retention of potassium. The work of Loeb (3), showing the so called salt effect as necessary for both the inward and outward diffusion of potassium from the fertilized eggs of *Fundulus*, does not apply to such a structure as a muscle cell. It does show that the presence of a certain amount of salt or of acidity or both is necessary

for the passage of potassium through the egg membrane and that sufficiently prolonged action of pure water makes this membrane very impermeable to potassium. But, as Loeb points out, the behavior of the hatched embryo is very different from that of the egg. In the egg we are dealing merely with the passage of potassium through a comparatively distinct membrane but in the case of most cells we must consider the taking up of potassium, whatever that means physicochemically, by the cell acting as a whole. Salts are always present and acidity absent in the medium bathing the great majority of living cells. Hamburger (4) has shown that blood corpuscles, washed with glucose solution, yield potassium to a potassium-free Ringer solution, and Jannink (5) has reported that potassium is given off by heart muscle during perfusion with a potassium-free solution. Howell and Duke (6) recorded a liberation of potassium from the heart perfused with Ringer solution during prolonged stimulation of the vagus. These and other experiments, though they point out interesting possibilities, do not reach the explanation of the conditions limiting the entrance or exit of potassium, considering cells in general.

Muscle seemed a favorable material to use because it gives an easily controlled range of physiological activity. The work of Lillie and of McClendon indicates that, under some circumstances at least, contracted muscles are more permeable than resting ones. Is this true for potassium? We sought the answer to the question by making determinations of the amount of potassium in frog muscles after perfusion with various solutions either with or without simultaneous excitation. The results have shown the great tenacity with which the cells hold potassium while bathed in a potassium-free Ringer solution irrespective of whether or not the muscles are made to contract. As soon, however, as the muscles are fatigued beyond physiological limits potassium diffuses out of the cells and as much as half of their store may be lost in about 5 hours. Since potassium is known to diffuse slowly from dead muscle and since extreme fatigue in excised muscle is an irreversible process this was to be expected. A study of the intermediate stage between mild activity and exhaustion, showed that a part, from 8 to 15 per cent, of the potassium may be removed by perfusion, either with or without stimulation, without

marked loss of irritability in the muscle substance. Our experiments though not including any quantitative measurements of irritability have shown to rough observation a progressive loss of excitability accompanying potassium depletion. This is to be expected from the well known contrast between the physiological effects of a potassium-free physiological saline and those of Ringer solution.

We have further undertaken to test the possibility that muscular contraction is favorable to the process by which potassium is absorbed. We have found that only a contracting muscle, in sharp contrast to a resting one, can take up rubidium and cesium, substances whose chemical properties are more like those of potassium than sodium, so as to retain them in the same sense that potassium is retained.

#### EXPERIMENTAL.

Large bull frogs weighing from 125 to 400 gm. were used. Some of them were collected in Louisiana in February, shipped in March and used for the experiments in April, May and June. Others were taken in Rhode Island or Massachusetts in summer and used shortly after collection. Perfusions were made through a glass canula in the dorsal aorta of the pithed animals. The muscles used for analysis were lightly drained on paper after removal from the animal and immediately weighed. After digesting the muscle in a mixture of nitric and sulphuric acids potassium determination was made by the method described by Clausen (7) for blood analysis. Muscles removed from frogs without experimentation, merely killing and bleeding the animal, gave results as shown in Table I.

The averages of these results, whether computed for the same muscles of different frogs or for the various muscles of the same frog, are fairly uniform and are in agreement with averages obtained by Fahr (8) and others, placing the normal content of potassium in frog muscle at 0.34 per cent. The considerable variation in the results is partly due to errors in sampling. The sartorius is too small in many frogs for a satisfactory analysis. The vastus cannot be removed intact in its sheath so as to yield clean cut prisms of muscle but is apt to suffer loss of some of its fluids during sampling. For this reason most of the experiments reported below were done with the gastrocnemius muscles which can be more satisfactorily sampled. Another

and more important cause of variation is due to fluctuations in the water content of the muscles. In a series of nineteen determinations of the water in fresh (not perfused) frog muscles the minimum was 79.10 per cent and the maximum 82.22 per cent. Comparison of these results with the previous history of the animals, season when collected, duration of captivity, etc., showed no consistent correlation. The variation is sufficient, however, to make it advisable, when comparing the potassium content of different muscles, to determine potassium as per cent of the dried weight of the muscle. The average for the series was 80.78 per cent which is within the range of the averages commonly given for water content of muscles of cold-blooded animals. A third, and chief reason, is the actual variation in the

TABLE I.

*Potassium Content of Fresh Normal Frog Muscle.  
Results Are in Per Cent of Potassium in Moist Muscle as Weighed.*

Muscle.	First frog.		Second frog.		Third frog.		Average.
	Left leg.	Right leg.	Left leg.	Right leg.	Left leg.	Right leg.	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Gastrocnemius.....	0.330	0.322	0.355	0.362	0.331		0.340
Sartorius.....	0.370	0.342	0.337	0.378	0.328	0.305	0.343
Vastus.....	0.328		0.300	0.327	0.348	0.349	0.330
Average.....	0.338		0.343		0.332		0.338

potassium content of the muscles of different frogs. For example the average of twelve analyses on eight frogs taken from localities in Rhode Island and Massachusetts during the summer and analyzed soon after collection was 1.807 per cent of potassium in the dried muscle while the average of seven analyses on muscles of six frogs brought from Louisiana in winter and used some months later was 1.591 per cent of potassium in dried muscle. The relation of the potassium content of frog muscle to reproductive activities, to sex, to the food supply, and to species would be interesting. Such studies have not been attempted in this work. The results indicate that a prolonged stay in an aquarium without food causes a diminution of potassium. The individual variations are greater than the analyti-

cal error and are due to something more than a mere seasonal variation since in a group of twelve analyses on eight summer frogs the maximum variation was 17 per cent of the average potassium content of the dried muscle while the analytical error as indicated by duplicate analyses of the same muscle or by analyses of the two fresh gastrocnemii, of the same frog did not exceed a maximum of 2.3 per cent and averaged 1.37 per cent.

The effect of perfusion with a potassium-free Ringer solution on the potassium content of muscle was contrasted with that of similar perfusion with Ringer solution. In each case both legs were perfused during 15 minutes with about 300 cc. of solution until the venous outflow appeared bloodless; the artery of one leg was then tied

TABLE II.

*The Potassium Content of Perfused Muscles.*

*Results Are in Per Cent of Potassium in the Moist Muscle, as Weighed.*

Muscle.	Perfused with potassium free solution.		Perfused with Ringer solution.	
	Slightly perfused. 300 cc.	Much perfused. 1800 cc.	Slightly perfused. 300 cc.	Much perfused. 1800 cc.
	<i>per cent</i> %	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Sartorius .....	0.263	0.240		
Vastus .....	0.275	0.222	0.292	0.248

off and 1500 cc. of solution perfused in  $1\frac{3}{4}$  hours through the other leg. Results are given in Table II.

The potassium content of the moist muscle was decreased in both experiments. Fahr (8) has shown that frog muscle immersed in Ringer solution during 20 hours contained a smaller percentage of potassium than fresh muscle, though after a similar immersion in isotonic sugar solution a greater potassium loss was shown. The lower per cent of potassium obtained after perfusion seemed attributable in part, at least, to the higher water content. This possibility was indicated by the obvious distention and edematous appearance of the much perfused leg. Edema as a result of perfusion with a number of physiological salt solutions has been observed by Guntzberg (9), Abel (10), and others. Another experiment was therefore

tried with determinations of water as well as potassium in the muscles. After perfusing 200 cc. of a potassium-free Ringer solution through both legs, one leg, was tied off and 800 cc. were then perfused in the course of 8 hours through the other leg. The gastrocnemius of the slightly perfused leg yielded 0.304 per cent of potassium and the same muscle of the other leg 0.267 per cent; but the first of these muscles contained 18.85 per cent of solids while the second had only 16.7 per cent, so that the slightly perfused muscle contained 1.61 per cent of potassium in its solids and the more extensively perfused one had 1.60 per cent of the solid matter. The average content of potassium in the solids of the fresh muscle is 1.75 per cent, so that muscles of both legs had doubtless lost some potassium. These experiments were done before the extent of variability in water and potassium content of muscles of different frogs was fully appreciated. In a further series samples of the gastrocnemius of one leg were taken after a brief perfusion, lasting less than 10 minutes, sufficient to remove all visible blood. The muscles of the other leg were then perfused during a suitable period and samples of both gastrocnemii, after vacuum desiccation to constant weight, were used entire for wet ashing and potassium titration. The effect on the perfused muscle was then computed as the percentage loss of the original potassium content as shown by the muscle that was not perfused. Such experiments gave consistent results and showed, as set forth in Table III, a loss of potassium during early stages of perfusion with the limit of potassium loss approached after 5 hours. Subsequently little or no loss of potassium occurs. The time of perfusion showed more consistent relation to potassium loss than did the amount of perfusion solution used. This indicates diffusion of potassium from the muscle cells into the fluid of the lymph spaces irrespective of considerable change in the rate at which the latter is replaced. Such a result may be explained by the fact that in any case the perfusion solution, present in such quantities as to greatly distend the lymph spaces, would be notably lower in potassium content than the intracellular fluid.

The relation of muscular activity to loss of potassium from the muscle cells was sought in the following experiment. Potassium-free Ringer solution was perfused through both legs during 2 hours.



500 cc. of solution were used. During the last  $1\frac{1}{2}$  hours the muscles of one leg were stimulated intermittently with strong induction shocks applied to the distal end of the severed lumbar plexus. The average potassium content of the muscles of the rested leg was then found to be 0.350 and of the stimulated muscle 0.306 per cent. In another experiment  $2\frac{1}{2}$  liters of solution were perfused and stimulation with intermittent rest periods was applied during  $2\frac{1}{2}$  hours to one leg. The average potassium content of the muscles of the rested leg

TABLE III.

*Loss of Potassium from Muscles Perfused with Potassium-free Ringer Solution.*

Total time of perfusion.	Total amount of solution used.	Average rate of perfusion.	Solids in control muscle.	Potassium in moist control muscle.	Potassium in solids of control muscle.	Solids in perfused muscle.	Potassium in moist perfused muscle.	Potassium in solids of perfused muscle.	Apparent loss of potassium.*
hours	cc.	cc. per minute	per cent	per cent	per cent	per cent	per cent	per cent	per cent
7	800	1.9	18.69	0.376	2.016	15.65	0.276	1.765	12.5
8	900	1.9	19.51	0.336	1.879	14.73	0.243	1.648	12.3
12	1800	2.5	18.87	0.335	1.775	16.20	0.245	1.511	14.8
$13\frac{2}{3}$	1500	1.8	18.96	0.335	1.765	14.25	0.214	1.499	15.1
$1\frac{1}{4}$	100	1.3	19.20	0.281	1.465	16.97	0.237	1.399	4.5
$5\frac{1}{3}$	800	2.4	20.60	0.292	1.417	17.97	0.233	1.298	8.3
$14\frac{1}{4}$	1000	1.2	18.75	0.313	1.670	14.64	0.225	1.537	8.5
$18\frac{3}{4}$	1600	1.4	18.56	0.309	1.667	15.48	0.237	1.532	8.1

\* Figures in this column are obtained, as explained in the text, by computing the difference between potassium in solids of control muscle and in those of perfused muscle as per cent of the potassium in the solids of the control muscle.

The frogs used in the first four experiments reported in this table were collected in summer and used shortly after they were brought to the laboratory. Those used in the last four experiments were collected in winter in Louisiana and used some months later.

was 0.299 per cent and of the stimulated leg 0.237 per cent. The stimulated muscles show lower potassium content than the resting ones. Siebeck's (11) observation, confirmed by Meigs and Atwood (12), that a muscle in isotonic potassium chloride solution takes on more weight if active than if at rest should be recalled in this connection. The apparent losses of potassium in the stimulated as contrasted with the rested muscles are not, indeed, greater than could be accounted for as percentage changes due to absorption of

water. We hoped to avoid this effect and produce more nearly physiological conditions by the use of gum arabic in the perfusion solution, employing, in short, a modified Bayliss solution. Gum arabic, purified by six alcohol precipitations from dilute hydrochloric acid solution, and showing by analysis an amount of potassium less than 0.01 per cent was used. The solution contained the following:

NaCl.....	6.5 gm.
CaCl <sub>2</sub> .....	0.25 "
NaHCO <sub>3</sub> .....	0.20 "
Gum arabic.....	45 "
Phenol-sulphonephthalein.....	a few drops.
Water.....	1 liter.

It had a pH of approximately 7.3; 700 cc. of the solution were perfused in  $6\frac{1}{2}$  hours through both legs. The muscles of the left leg were stimulated through the lumbar plexus with maximal tetanizing induction shocks lasting 1 second each, given by an automatic stimulator at 30 second intervals during the first half of each hour with half hour rest periods intervening. These muscles, then, made 360 contractions lasting 1 second each. The muscles of both legs showed, at the end of the experiment, good though not equal irritability. The average potassium content of the muscles of the stimulated leg was 0.229 per cent, of the muscles of the other leg, 0.228 per cent. Both of these figures are lower than the percentages of potassium found in normal frog muscle. Both legs showed, however, some swelling in spite of the gum arabic. To determine the effect of contraction on the potassium content it seemed necessary to compute it in relation to the solids of the muscle. A further experiment for this purpose was undertaken. 900 cc. of potassium-free Ringer solution were perfused through both legs in  $8\frac{1}{2}$  hours. Gum arabic because of the labor involved in making it potassium-free was not used. The muscles of one leg, stimulated by the method described for the preceding experiment, gave 480 contractions, of 1 second each. The muscles of both legs showed good irritability at the end of the experiment. The average potassium content of the muscles of the stimulated leg was 0.203 per cent, of the muscles of the other leg, 0.230 per cent, but computed as percentages of the solids: 1.86 per cent for the former and 1.85 per cent for the latter. No especial loss of potassium due to stimulation was shown.

In addition, the experiment reported on the second line in Table III included stimulation by the same method so that the muscles in the course of 8 hours of perfusion gave 330 contractions of 1 second each. The results of this experiment do not fall out of line with those including no stimulation. Results therefore give no indication of loss of potassium while the muscle is contracting, other than the loss attributable to the presence of a potassium-free medium.

Such results are in marked contrast to those obtained when muscle is stimulated enough to cause loss of irritability. The considerable difference between the potassium content of the rested and the exhausted muscle as found in the following experiment seemed too great to be explained on the basis of change in muscle weight. Both legs were perfused with potassium-free Ringer solution during 2 hours. The muscles of one leg were given prolonged tetanizing stimuli at first through the nerves until they failed to respond and then by direct stimulation until exhausted. The average potassium content of the muscles of this leg was 0.141 per cent and that of the rested muscles, 0.266 per cent. A similar experiment to determine the potassium content of the muscle *solids* after exhaustive stimulation was made. 1600 cc. of potassium-free Ringer solution were perfused through both legs in  $6\frac{1}{4}$  hours. The muscles of one leg were given direct tetanizing stimuli for varying periods with intervening rest. The total time of stimulation was 98 minutes. The average potassium content of the muscles of this leg was 0.102 per cent; of the others, 0.221 per cent. Computed as percentages of the solids there was 0.89 per cent in the fatigued and 1.70 per cent in the rested muscles. The stimulated muscles lost about half of their potassium.

The development of acidity in a fatigued muscle suggests that hydrogen ion concentration is a factor in the control of the processes by which potassium is retained or set free in the cells. Experiments to test this possibility will be the subject of a later communication. For the experiments reported in this paper perfusion solutions were in general adjusted with sodium bicarbonate, using Clark and Lubs (13) indicators, to a pH of approximately 7.3.

That muscular contraction does enable the cells to *take in* potassium is indicated by experiments on the absorption by muscles of rubidium and cesium, substances very similar to potassium in chemical and

physiological behavior. This work is reported in another paper (14), but may be briefly summarized in so far as it applies to the subject under discussion. It included experiments in which frog's legs were perfused with a Ringer solution whose potassium chloride was replaced by equivalent amounts of rubidium or cesium chloride. The muscles of one leg were meanwhile stimulated to contract without complete fatigue. Subsequently both legs were perfused with a potassium-free Ringer solution until the outgoing fluid gave no spectroscopic test for rubidium or cesium. The muscles of both legs were then analyzed for rubidium or cesium. Only those of the leg which had been stimulated during the first perfusion showed the presence of these metals. To take them, and therefore their close homologue, potassium, into the muscle in such manner as to be retained, in a non-diffusible form would seem to require contractile activity of the muscle.

#### DISCUSSION.

These experiments indicate that, as Loeb (1) has suggested, the potassium of muscle cells can be considered as existing in two different states. The portion constituting one of them is comparatively mobile, consists of about 15 per cent of the muscle potassium in summer frogs and a smaller proportion in winter frogs, and can be removed from frog muscle, under the conditions described, by mere perfusion with a potassium-free Ringer solution. The rate of its loss seems to be unaffected by contractile activity of the muscle. The remainder of the potassium, amounting to approximately 15 per cent of the dry solids of a perfused muscle, markedly resists outward diffusion but is liberated and rapidly lost when the muscle is stimulated to an extreme stage of fatigue. That some deep-seated change in the chemical composition of a muscle occurs during unusually prolonged exertion is evident from the long duration of the after effects. That the change causes an alteration of the conditions governing the partition of inorganic constituents between dissociable and non-dissociable forms is suggested by these observations. The greater osmotic pressure of fatigued muscle as compared with non-fatigued was shown by Moore (15) and taken to indicate an increased concentration of electrolytes in the cell during contraction unless they are removed by the circulation. The work of Loeb (16) showing the absolute

dependence of the formation of metallic salts of proteins on hydrogen ion concentration is helpful in forming a conception of the behavior of potassium. If one believes with Robertson (2) that salts of the proteins may be so built that the inorganic constituent is, partly at least, in non-dissociable form then one can readily conceive of how an intracellular change of hydrogen ion concentration such as occurs in a fatigued muscle would markedly alter the distribution of potassium, and doubtless other inorganic constituents, between the dissociable and the non-dissociable forms.

As to the absorption of potassium a conception of how contraction might be essential to the mechanism involved is difficult. One notes the generally accepted idea that ion movements having definite relation to membranes are concerned in excitation. This idea is, of course, the basis of the Nernst theory of stimulation and is assumed in the conception of the mode of propagation of excitation as developed by Lillie (17). Because ions move during excitation and perhaps penetrate membranes, are we to assume that the potassium ion can penetrate the cell only at that time? In the light of our present knowledge it seems necessary to believe that cell permeability is not merely a matter of passage through an external membrane but that the physiological behavior of the protoplasm as a whole must be considered. The work of Wishart (18) on the distribution of glucose between plasma and corpuscles may be cited as one example of such observations. If contraction, involving migration of ions, causes a sort of fixation of rubidium or cesium within the cell it is fair to suppose that potassium would be similarly affected. That potassium favors excitability by its presence at cell surfaces has been amply demonstrated for a variety of tissues, so that some significant movement of potassium ions is involved, among other things, in a response such as muscular contraction. Our results suggest that movement at the cell surface is not the only factor concerned but that some transformation of potassium into a non-dissociable form occurs, presumably within the protoplasm. In this connection the work of Crozier (19) on sensory activation of cells by acids is of interest since it led him to the view that stimulation does not merely involve an increase in permeability but that the change of condition of materials at the surface of cells is instrumental in determining diffusion of ions within the cell.

## SUMMARY.

1. Individual variations in the potassium content of the fresh muscles of frogs are notable even when computed as percentages of the dry solids. The potassium content averaged higher in freshly collected summer frogs than in winter frogs after a period of captivity.

2. Muscles show a loss of from 8 to 15 per cent of their potassium during perfusion with potassium-free Ringer solution but tenaciously hold the remainder.

3. Muscles, stimulated to contract under conditions that do not produce irreversible stages of fatigue, show losses of potassium no greater than those attributable to the presence of a potassium-free medium.

4. A condition favorable to the taking up of potassium probably occurs in a contracting muscle because rubidium and cesium, substances very similar to potassium in chemical and physiological behavior, are absorbed in retainable form by a contracting muscle but not by a resting one.

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# COMPARATIVE HYDROLYSIS OF GELATIN BY PEPSIN, TRYPSIN, ACID, AND ALKALI.

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Gelatin and proteins in general may be hydrolyzed by either acids (hydrogen ions), alkali (hydroxyl ions) pepsin, or trypsin. In all cases the reaction consists in the rupturing of one or more of the peptide linkings with the addition of 1 molecule of water. The ease with which the various linkages are split is very different, as was shown by Fischer,<sup>1</sup> in his study of the hydrolysis of the polypeptids. The same phenomenon is shown by the fact that the products of partial hydrolysis of the proteins form a series of increasing complexity—a result of the fact that some of the linkages are much more easily split than others. The same fact is brought out by following the course of the reaction in strong acid for example. If all the linkages were split at the same rate, the reaction would follow the course of a monomolecular one. This is not the case. The velocity decreases steadily as the reaction proceeds, showing that some of the linkages are split more easily than others.

The question arises then as to whether those linkages which are most easily split by acid, for instance, are also the most easily split by pepsin and trypsin. There is evidence already that this is not the case. Henriques and Gjaldbäk<sup>2</sup> have shown by an ingenious modification of the formol titration that protein solutions having the same total content of titratable amino or carboxyl groups but which have been brought to this stage by different hydrolytic agents, may be distinguished from each other by their behavior when titrated with alkali to different end points, showing that, although the total

<sup>1</sup> Fischer, E., *Untersuchungen über Aminosäuren Polypeptide und Proteine*. Berlin, 1906.

<sup>2</sup> Henriques, V., and Gjaldbäk, J. K., *Z. physiol. Chem.*, 1911, lxxv, 363; 1913, lxxxiii, 83.

number of linkages split was the same in all the solutions, different ones had been split in each case. They found that the course of the reaction was similar with pepsin and acid, and with trypsin and alkali. The study of the hydrolytic products of trypsin and pepsin leads to the same conclusion.<sup>3</sup> It is usually stated that trypsin hydrolysis leads to more amino-acid formation.

More quantitative evidence may be obtained, however, by a slightly different mode of procedure; namely, by adding the enzyme in question to a solution of the protein which has already been partially hydrolyzed by some other means and noting the subsequent increase in hydrolysis. If the two reactions follow the same course, *i.e.*, if the same linkages are split in both cases, the final amount of hydrolysis will be the same irrespective of the stage at which the enzyme is added. If, however, the preceding hydrolysis has split the protein at a linkage which is not attacked by the enzyme, then the total amount of hydrolysis will be the greater the farther the hydrolysis had proceeded before the enzyme was added. That is, the total hydrolysis will be equal to the sum of the enzyme hydrolysis (on the unhydrolyzed protein) plus that which had taken place due to the other hydrolytic agent. Since it is known that acids and alkali are capable of hydrolyzing the proteins to their constituent amino-acids, it is obvious that they must be able to split all the linkages so that a stage must eventually be reached in acid or alkali hydrolysis at which the addition of the enzymes will cause no further hydrolysis. The stage in the hydrolysis at which this occurs will evidently be a measure of the difference in the course of the two reactions. If the linkages which are most easily split by pepsin, for instance, are the ones which are most slowly attacked by acid, it will evidently be necessary to almost completely hydrolyze with acid the protein before reaching a stage at which the addition of pepsin will cause no further increase. As will be seen below, this is really the case.

The results are complicated by the fact that the ease of hydrolysis depends on other factors as well as on the particular linkage which is hydrolyzed. This was shown by Fischer<sup>1</sup> who found that tetraglycyl glycine may be hydrolyzed by trypsin whereas triglycyl glycine is not

<sup>3</sup> Cf. Fischer.<sup>1</sup>



hydrolyzed. It is, therefore, not possible to conclude that the enzyme ceases to act, when a certain stage of the hydrolysis has been reached, because all the linkages which it is capable of hydrolyzing have already been split. The molecule may have been modified in some other way and so become resistant to the action of the enzyme although still containing a linkage which under certain conditions may be attacked. The same influence is undoubtedly also shown on the rate of hydrolysis by acids or alkali. There does not seem to be any evidence to distinguish qualitatively between the specificity of an enzyme and of hydrogen ions.

The procedure outlined above was followed in the experiments described in this paper. The gelatin was hydrolyzed to various stages by one of the hydrolyzing agents mentioned, pepsin or trypsin then added, and the increase in hydrolysis noted. The reaction was followed by means of a slight modification of the formol titration.

#### EXPERIMENTAL.

*Gelatin.*—Powdered gelatin, purified by washing at the isoelectric point,<sup>4</sup> was used in all the experiments. The solutions were made up to contain 5 gm. of gelatin per 100 cc.

*Pepsin.*—The pepsin used was an active preparation of Fairchild Bros. (U. S. P. 1/19,000). It was prepared for use by dissolving 5 gm. in 100 cc. of water, adjusting to pH 2.5 with HCl and dialyzing under pressure against 0.01 N HCl for several days. The solution thus obtained had a very low titration which remained constant. In the concentration used the correction for the pepsin solution was negligible. The solution is referred to as 5 per cent pepsin.

*Trypsin.*—Fairchild's trypsin was used. It was prepared for use by dissolving 10 gm. in 100 cc. of water and dialyzing under pressure against running tap water at 6°C. for about 18 hours. The solution is very unstable and loses its activity in 3 or 4 days even at 2°C. The formol titration of this solution was negligible in the concentrations used and remained constant. This solution is referred to as 10 per cent dialyzed trypsin.

*Hydrogen ion determinations.*—The determinations were made by the E. M. F. method.

*Formol titration.*—The titration was carried out as described in a previous paper.<sup>5</sup> The method consists essentially in freeing the solutions from CO<sub>2</sub> by adjusting the pH with acid, and boiling for a few seconds. The solution is then

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<sup>4</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 237.

<sup>5</sup> Northrop, J. H., *J. Gen. Physiol.*, 1920-21, iii, 715.

accurately adjusted to pH 7.0, using neutral red as an indicator. The formaldehyde is then added and the titration carried out as usual with 0.1 N NaOH, using thymol blue as indicator and an end-point of about 8.2. The titration was made on 10 cc. until the titration figure became more than 5 cc., after which 5 cc. were analyzed. The results have been calculated on the basis of 10 cc. of 5 per cent gelatin. The titration value for this concentration of gelatin was found to be 2.0 cc. The complete hydrolysis of gelatin increases the amino nitrogen (or formol titration) about 20 times.<sup>5, 6</sup> The percentage total hydrolysis may therefore be found from the figures by dividing by 40.0.

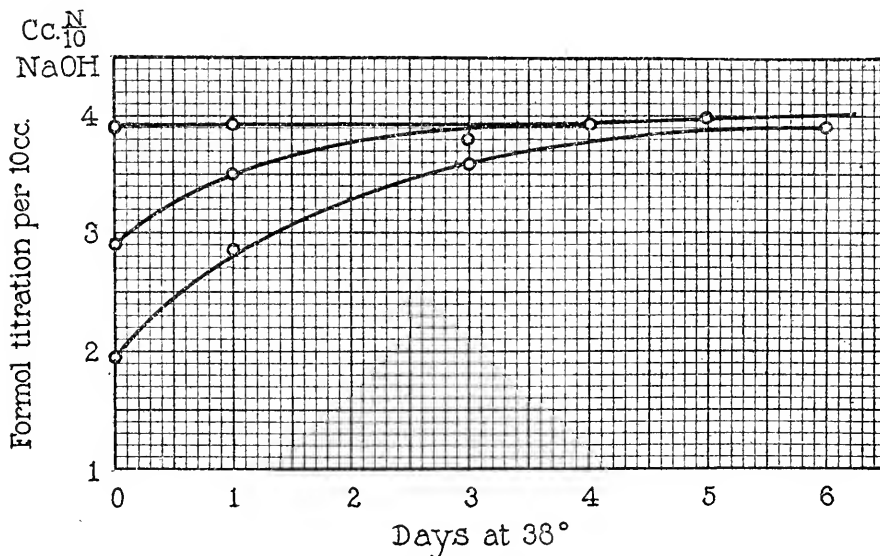


FIG. 1. Hydrolysis of gelatin by pepsin. 500 cc. of solution containing 25 gm. of gelatin, 20 cc. of 5 per cent dialyzed pepsin, and 40 cc. of 1.0 N HCl placed at 38°C. (pH = 2.2). 50 cc. samples pipetted out after 0, 1, and 4 days, and kept at 3° until all had been taken. 5 cc. of 5 per cent pepsin added to each sample and the samples placed at 38°C. Formol titration was run on 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

#### *Hydrolysis by Pepsin Alone.*

The results of this experiment are given in Fig. 1. The figure shows that under the conditions of this experiment, the pepsin is able to double the formol titration of the gelatin (an increase of 2 cc. per 10 cc. of 5 per cent gelatin); *i.e.*, the number of free carboxyl groups

<sup>6</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1911-12, **x**, 49.

is doubled. Since in completely hydrolyzed gelatin the free carboxyl groups (or the amino nitrogen) is increased about twenty times, the hydrolysis due to pepsin alone corresponds to about 5 per cent of the total hydrolysis. It will be seen from the figure that this result is independent of the amount of pepsin added and also of the stage of hydrolysis at which the pepsin is added. It is not possible to say, however, whether or not this really represents the complete action of the pepsin, since the hydrolysis is still continuing slowly and if the analyses were made at weekly intervals it would be found that the values were increasing. This slow increase, however, approaches asymptotically the increase due to the acid alone, so that it is impossible to say when the action of the pepsin stops. The real end-point for pepsin digestion could be definitely fixed only by reaching the same value from both sides. For the purpose of these experiments it is permissible to take the end-point as that point at which the addition of more pepsin causes no further hydrolysis in the course of 3 or 4 days (the action of the acid alone in this time is within the limits of error of the method). In the experiment just described this value evidently corresponds to a titration of 4 cc. of 0.1 N NaOH per 10 cc. of 5 per cent gelatin.

*Action of Pepsin on Gelatin Partially Hydrolyzed by Trypsin.*

The results of this experiment are shown in Fig. 2. The experiment shows that the increased hydrolysis due to the pepsin becomes less the farther the hydrolysis due to the trypsin has been carried. The linkages which are split by pepsin are also evidently attacked by trypsin. At the same time, however, the trypsin is also hydrolyzing some linkages which are not attacked by pepsin. This is shown by the fact (Table I) that the addition of pepsin to a solution which has been hydrolyzed by trypsin to a titration of 7.0 cc. causes a still further increase, although, as we have seen, pepsin alone can only carry the hydrolysis to 4.0 cc. The same result was obtained by Henriques and Gjaldbæk<sup>2</sup> with other proteins.

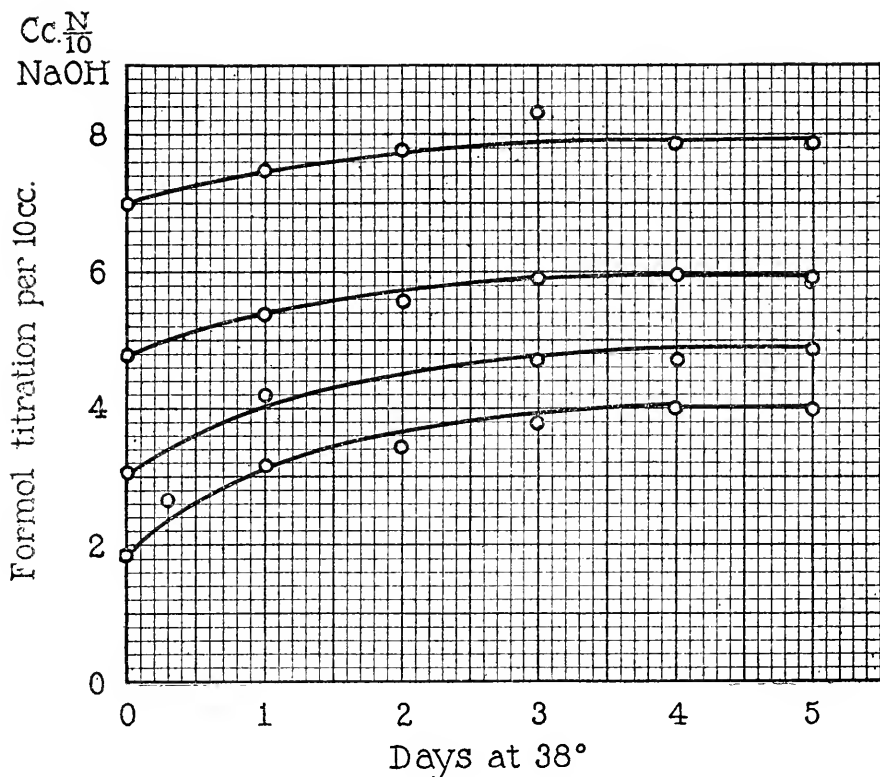


FIG. 2. Action of pepsin on gelatin previously partially hydrolyzed by trypsin. 500 cc. of 5 per cent gelatin containing 25 cc. of 1.0 M  $\text{Na}_2\text{CO}_3$  placed at  $38^\circ\text{C}$ . and 5 cc. of 10 per cent dialyzed trypsin solution added ( $\text{pH} = 9.5$ ). 25 cc. pipetted out at intervals and 2 cc. of 4.2 M HCl added ( $\text{pH} = 1.8$ ), and the samples kept at  $3^\circ$  until all had been taken. 5 cc. of 5 per cent pepsin then added to each sample and the samples replaced at  $38^\circ$ . Formol titration was run on 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE I.

*Addition of Pepsin to Gelatin Partially Hydrolyzed by Trypsin.*

	Formol Titration per 10 cc. of Gelatin Solution.			
Before addition of pepsin.....	1.9	3.1	4.8	7.0
Increase in titration due to action of pepsin..	2.1	1.9	1.1	0.9

*Action of Pepsin on Gelatin Partially Hydrolyzed by Alkali.*

The results of this experiment are given in Fig. 3 and Table II. The results are very similar to those obtained with trypsin and pepsin. The linkages attacked by pepsin are evidently quite rapidly attacked by the alkali although some are still left after the hydrolysis has reached titration value of at least 10 cc.

*Action of Pepsin on Gelatin Partially Hydrolyzed by Acid.*

These experiments are summarized in Fig. 4 and Table III. They show that the linkages attacked by pepsin are among the most resistant to the action of acid since the addition of the pepsin caused the normal increase of 2 cc. even after the acid hydrolysis had proceeded to a value of over 6.0 cc. There is no evidence of a true equilibrium in the presence of pepsin since no *decrease* is noted at any time even when the acid hydrolysis has been carried far beyond the end-point reached with pepsin acting on the unhydrolyzed protein. Such a reverse or synthetic action of pepsin has occasionally been recorded (the so called *plastein formation*<sup>7</sup>) in the case of other proteins. It is apparently always connected with the formation of a precipitate. This fact makes it appear possible that the decrease in the titration value is due to the formation of some insoluble compound from substances present in the pepsin and protein solutions. This decrease has not been noted in the case of gelatin solutions and these are the ones in which no precipitate forms. The experiments described here show that the hydrolysis by pepsin follows quite a different course from that followed by acid hydrolysis. The products formed must therefore be different. It seems unlikely that the pepsin could have any synthetic action on a solution containing substances which differ from those formed by the action of pepsin itself.

*Hydrolysis by Trypsin.*

The extent of the hydrolysis by trypsin alone is shown in Fig. 5. The hydrolysis continues until a titration value of about 15 cc. is reached. The same uncertainty as to whether this is the real end-point evidently exists here just as in the pepsin hydrolysis.

<sup>7</sup> Henriques, V., and Gjaldbæk, J. K., *Z. physiol. Chem.*, 1911, lxxi, 485; 1912, lxxxi, 439.

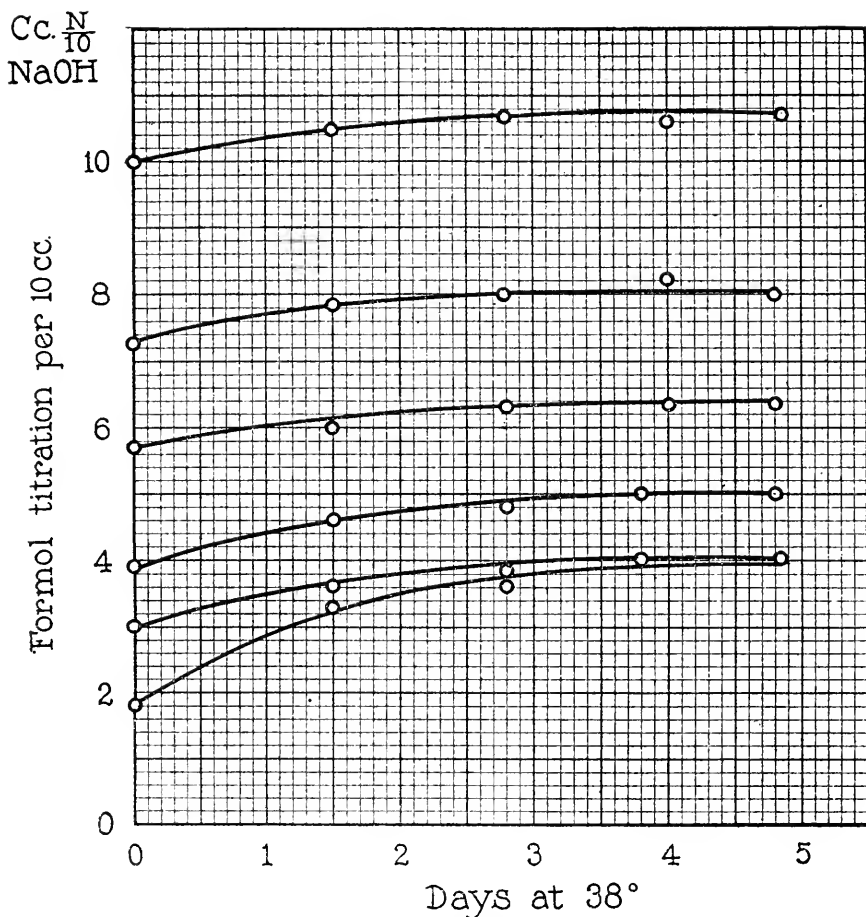


FIG. 3. Action of pepsin on gelatin partially hydrolyzed by alkali. 500 cc. of 10 per cent gelatin containing 8 cc. of 3 N NaOH kept at about 80°C. 50 cc. pipetted out at intervals and added to 50 cc. of 0.3 N HCl (pH = 2.0), these samples kept at 2° until all had been taken and 5 cc. of 5 per cent pepsin then added to each, and the samples placed at 38°. Formol titration was run on 5 or 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE II.

*Addition of Pepsin to Gelatin Partially Hydrolyzed by Alkali.*

	Formol Titration per 10 cc. of Solution.					
	1.9	3.0	3.9	5.7	7.3	10.0
Before addition of pepsin.....	1.9	3.0	3.9	5.7	7.3	10.0
Increase due to action of pepsin.....	2.0	1.0	1.1	0.7	0.7	0.6

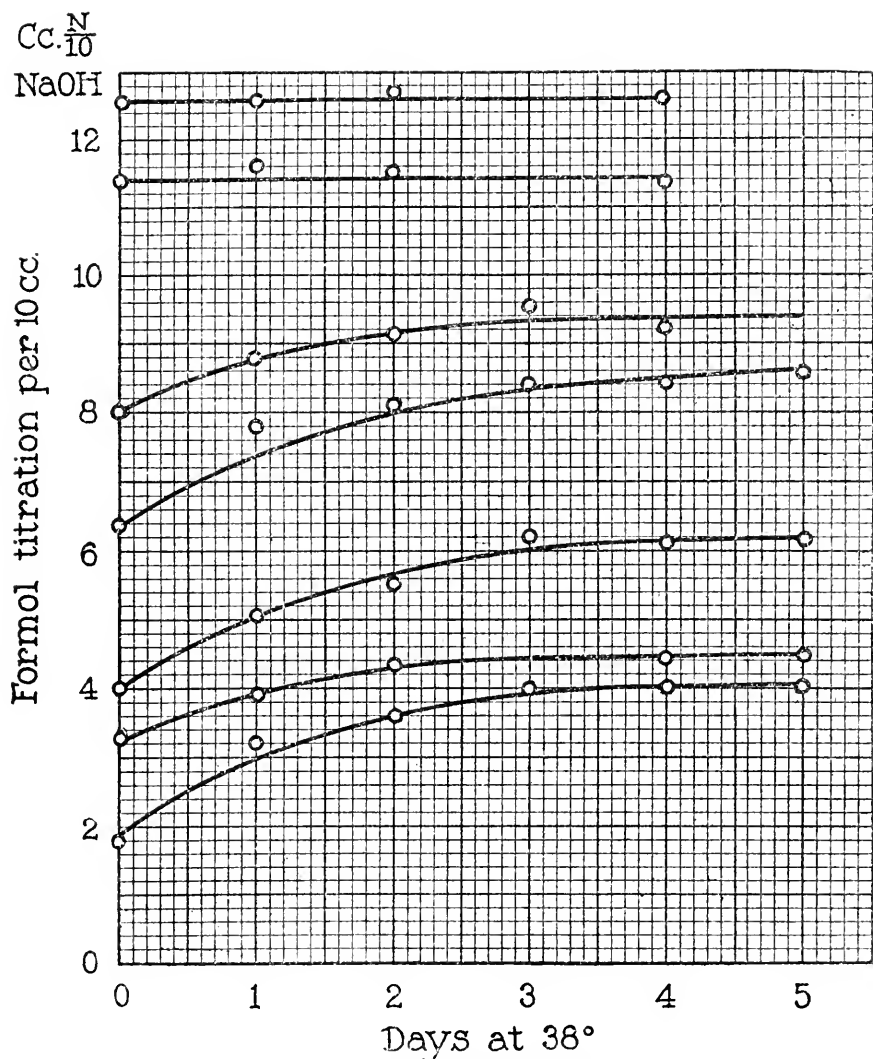


FIG. 4. Action of pepsin on gelatin partially hydrolyzed by acid. 500 cc. of 5 per cent gelatin containing 12 cc. of 4.0 M HCl (pH 1.8) kept at 38°. pH kept constant by addition of more HCl from time to time. 50 cc. samples pipetted out at intervals of 2 days and kept at 3° until all had been taken. 5 cc. of 5 per cent pepsin then added and the samples replaced at 38°. Formol titration was run on 5 or 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE III.

*Addition of Pepsin to Gelatin Partially Hydrolyzed by Acid.*

	Formol Titration per 10 cc. of Solution.						
	1.9	3.3	4.0	6.4	8.0	11.4	12.6
Before addition of pepsin.....	1.9	3.3	4.0	6.4	8.0	11.4	12.6
Increase due to pepsin.....	2.1	(1.2)	2.0	2.0	1.0	0.0	0.0

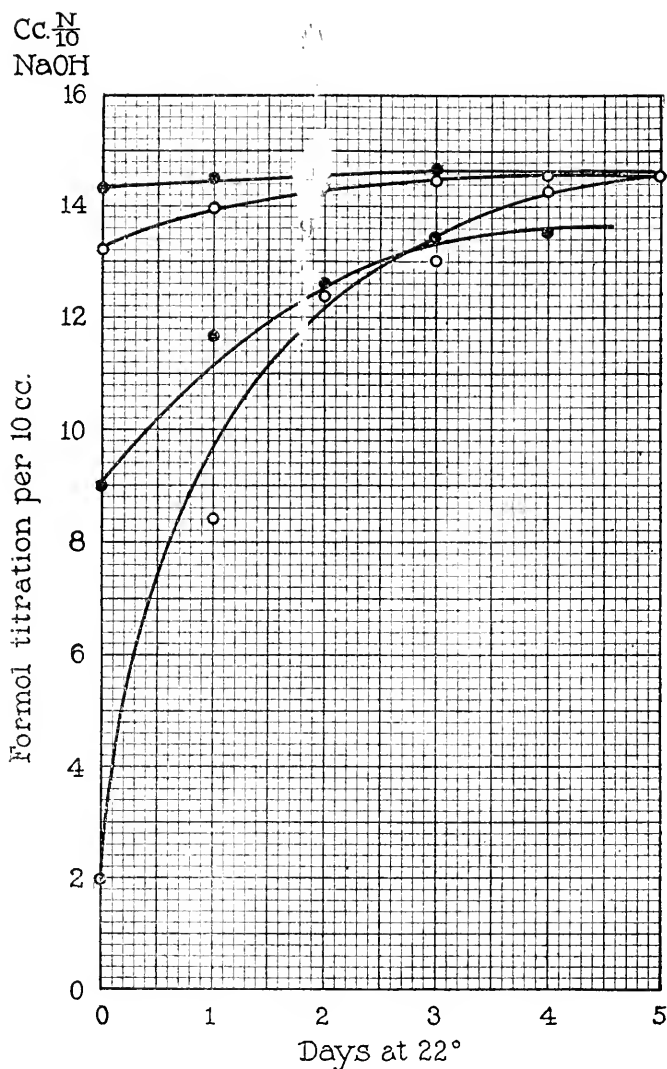


FIG. 5. Hydrolysis of gelatin by trypsin alone. 8 cc. of 10 per cent trypsin were added to 200 cc. of 5 per cent gelatin containing 10 cc. of 1.0 M  $\text{Na}_2\text{CO}_3$ , and the solution kept at 22°. 25 cc. samples pipetted out at intervals and kept at 3°C. until all had been collected. 1 cc. of 10 per cent trypsin was then added to each sample, the bottles placed at 22°, and formol titration was run on 5 cc., as shown in the figure. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.



*Action of Trypsin on Gelatin Partially Hydrolyzed by Pepsin.*

The results of this experiment are shown in Fig. 6. The final point reached by the trypsin hydrolysis is independent of the amount of hydrolysis previously accomplished by the pepsin. The linkages hydrolyzed by pepsin are therefore evidently also hydrolyzed by trypsin so that it is immaterial whether the pepsin acts on the protein or not, as far as the final stage reached is concerned. It is possible that the *rate* of hydrolysis may be greater if the pepsin acts first since Fig. 2 showed that those linkages attacked by pepsin are among the most resistant to the action of trypsin. The experiment confirms the result shown in Fig. 2, that trypsin hydrolyzes the same linkages as does pepsin but also attacks others which are split slowly if at all by pepsin.

TABLE IV.

*Addition of Trypsin to Gelatin Partially Hydrolyzed by Alkali.*

	Formol Titration per 10 cc. of Solution.			
Before addition of trypsin.....	2.0	4.9	9.6	13.0
Increase due to trypsin.....	8.6	4.8	0.7	0.0

TABLE V.

*Addition of Trypsin to Gelatin Partially Hydrolyzed by Acid.*

	Formol Titration per 10 cc. of Gelatin.			
Before addition of trypsin.....	2.0	6.7	10.2	18.0
Increase due to trypsin.....	8.2	4.5	5.4	5.0

*Action of Trypsin on Gelatin Previously Hydrolyzed by Alkali.*

The results of this experiment are given in Fig. 7 and Table IV. Alkali hydrolysis evidently follows very nearly the same course as does trypsin hydrolysis since the increased hydrolysis due to the addition of trypsin becomes rapidly less and stops when the alkali hydrolysis has reached about 13 cc. This corresponds to the point reached by trypsin alone. The linkages split must therefore be the same in both cases. In this case also no evidence of a reversal of the action was observed although the experiment was carried much further than is shown in the figure.

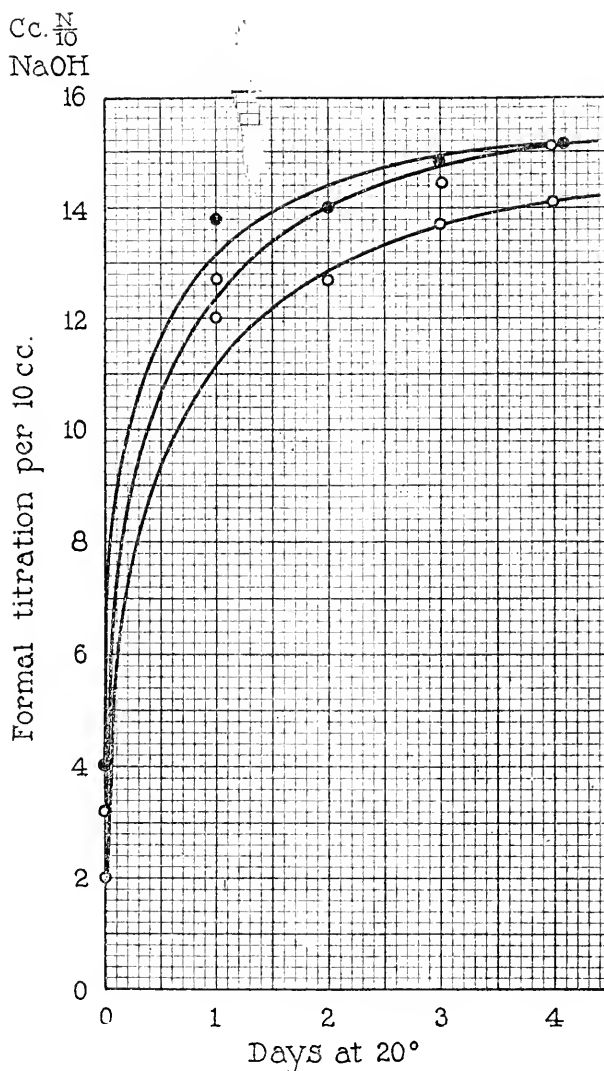


FIG. 6. Action of trypsin on gelatin partially hydrolyzed by pepsin. 500 cc. of 5 per cent gelatin solution brought to pH 2.2 with HCl, 20 cc. of 5 per cent pepsin solution added and the solution kept at 38°C. 25 cc. samples pipetted out at intervals, 5 cc. of 1.0 M  $\text{Na}_2\text{CO}_3$  added and the samples kept at 3° until all had been collected. 1 cc. of 10 per cent dialyzed trypsin then added and the samples placed at 22°. Formol titration was run on 5 cc. samples. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

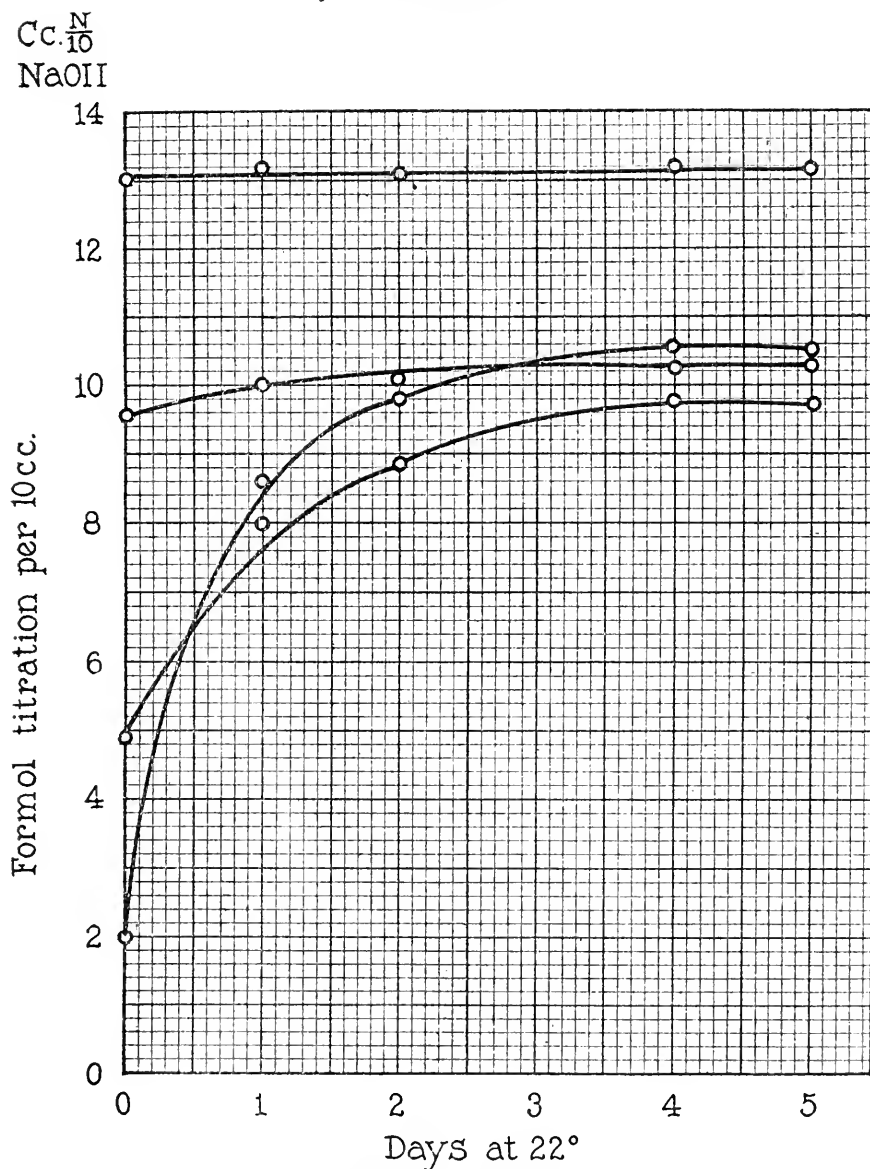


FIG. 7. Action of trypsin on gelatin partially hydrolyzed by alkali. 500 cc. of 5 per cent gelatin solution containing 50 cc. of 1.0 M  $\text{Na}_2\text{CO}_3$  kept at  $90^\circ\text{C}$ . 25 cc. samples pipetted out at intervals, and kept at  $3^\circ$  until all had been collected. 1 cc. of 10 per cent dialyzed trypsin then added to each sample and the solutions kept at  $22^\circ$ . Formol titration was run on 5 cc. The figures plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

$\text{Cc. } \frac{N}{10}$   
 $\text{NaOH}$

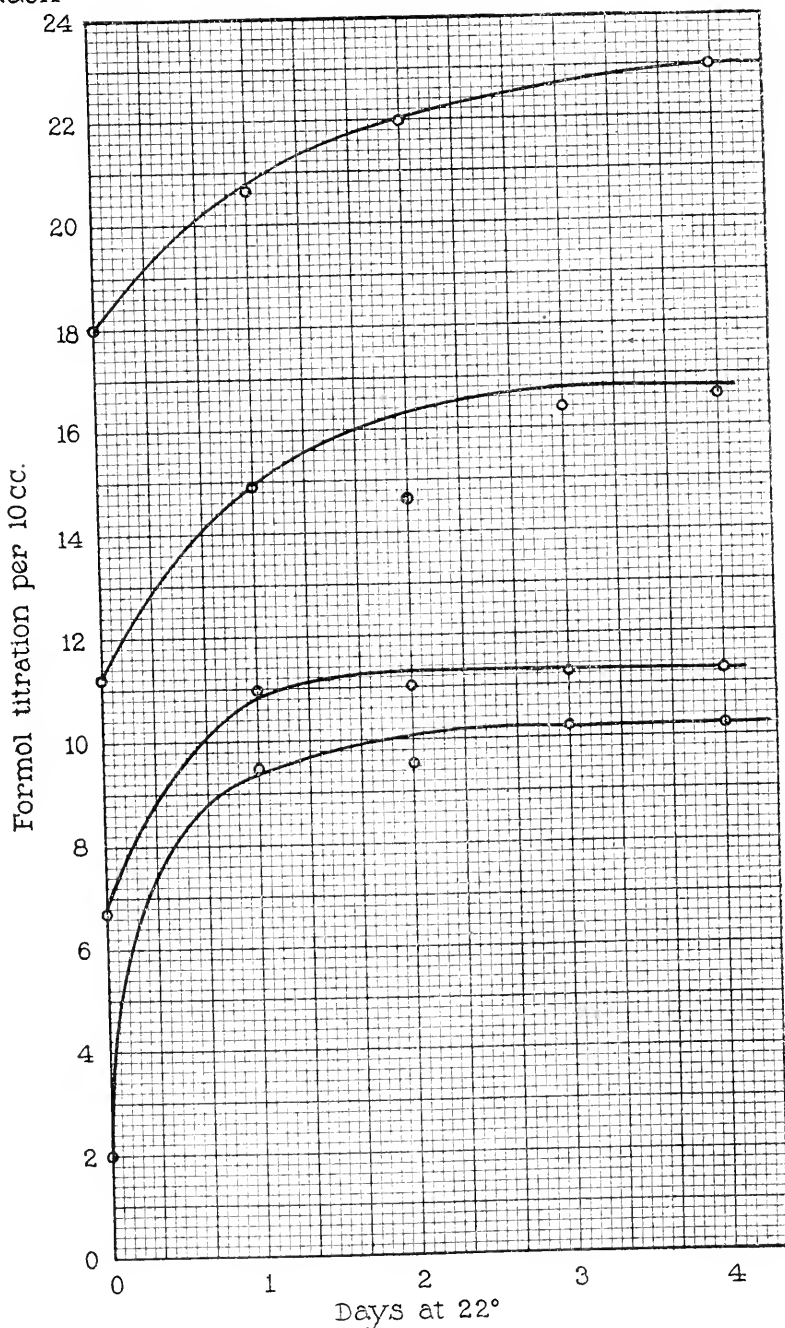


FIG. 8. Action of trypsin on gelatin partially hydrolyzed by acid. 500 cc. of 5 per cent gelatin brought to pH 1.8 with HCl and the solution kept at 90°C. 25 cc. samples pipetted out at intervals and 5 cc. of 1.0 M  $\text{Na}_2\text{CO}_3$  added to each. Samples kept at 3° until all had been collected. They were then put at 22° and 1 cc. of 10 per cent dialyzed trypsin added to each sample. Formol titration was run on 5 cc. The figures plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

*Action of Trypsin on Gelatin previously Hydrolyzed by Acid.*

The results of this experiment are shown in Fig. 8, and Table V. The course of the acid hydrolysis is quite different from that due to the trypsin since the addition of trypsin to the hydrolyzed gelatin is able to cause a still further increase even after the total number of linkages already split by the acid is greater than that which could be split by trypsin alone.

## SUMMARY.

A comparison has been made of the relative velocity of hydrolysis of the various peptid linkings of the gelatin molecule when hydrolyzed by acid, alkali, pepsin or trypsin. It has been found that:

1. Those linkages which are most rapidly split by pepsin or trypsin are among the more resistant to acid hydrolysis.
2. Those linkages which are hydrolyzed by pepsin are also hydrolyzed by trypsin.
3. Trypsin hydrolyzes linkages which are not attacked by pepsin.
4. Of the linkages which are hydrolyzed by both enzymes, those which are most rapidly hydrolyzed by pepsin are only slowly attacked by trypsin.
5. Those linkages which are attacked by trypsin or pepsin are among the ones first (most rapidly) hydrolyzed by alkali.

In general it may be said that the course of the early stages of hydrolysis of gelatin is similar with alkali, trypsin, or pepsin and quite different with acid.



# DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

## IV. VISCOSITY—*Continued.*

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1. In order to complete the demonstration that the viscosity of suspensions of powdered gelatin in water is influenced by electrolytes in a similar way as the viscosity of solutions of gelatin, it is necessary to prove that the characteristic valency effect exists also in the case of the viscosity of suspended particles of solid gelatin in water.

This proof is furnished in Fig. 1. 0.5 gm. of finely powdered gelatin (going through a sieve of mesh size 100 but not through a sieve of mesh size 120) of pH 7.0 was put into a series of beakers containing each 100 cc. of HCl of different pH and kept in the solution overnight at a temperature of 20°C. Simultaneously a similar series of beakers containing each 100 cc. of  $H_3PO_4$  and  $H_2SO_4$  of different pH (instead of HCl) were prepared, each receiving also 0.5 gm. of powdered gelatin. After 19 hours the viscosities of all these series of suspensions were determined at 20°C. Fig. 1 gives the result, the ordinates being the values for the viscosity ratios, gelatin suspension: water, and the abscissæ are the pH of the solid gelatin particles at equilibrium. The curves show that the viscosity of suspensions of gelatin sulfate is a little less than half that of suspensions of gelatin chloride and phosphate of the same pH. The curves for the suspensions of gelatin chloride and gelatin phosphate are alike, with the exception of part of the descending branch.

Experiments on the influence of these three acids on swelling published in a previous paper<sup>1</sup> show that the curves for the relative volume of powdered gelatin in solutions of these three acids are similar to the

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 253.

viscosity curves in Fig. 1 of this paper since the relative volume of gelatin sulfate was found to be not far from one-half of that of gelatin chloride or gelatin phosphate of the same pH. This demonstration completes the proof that the viscosity of *suspensions* of powdered gelatin in water of different pH is influenced in the same way by

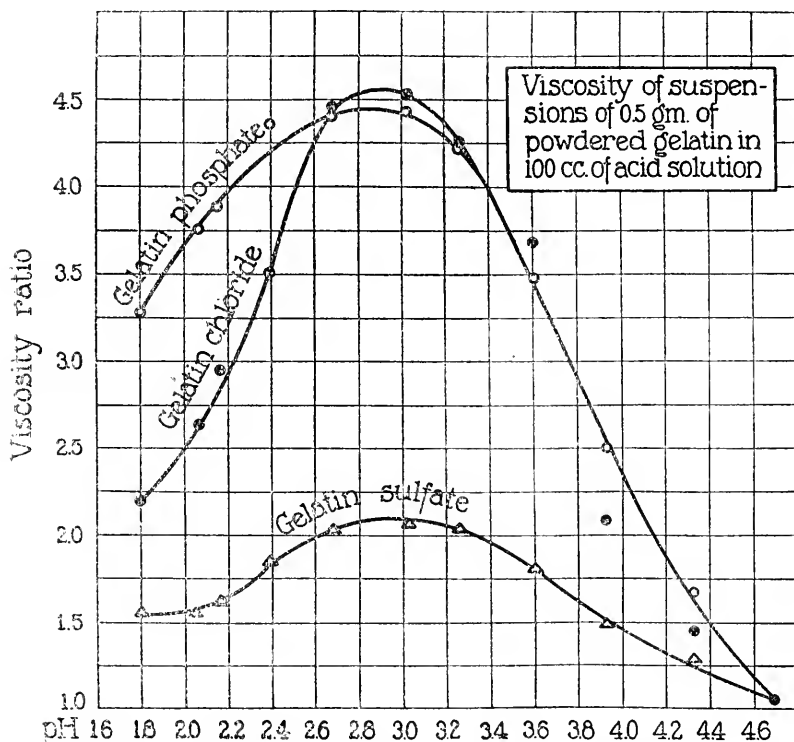


FIG. 1. Viscosity of suspensions of 0.5 gm. of powdered gelatin of grain size 100 to 120. Abscissæ are the pH, the ordinates the ratio of time of outflow of suspension: time of outflow of water. The influence of HCl and  $H_3PO_4$  is practically identical for the same pH while  $H_2SO_4$  depresses the viscosity of the suspensions to a little less than one-half of that for HCl.

electrolytes as is the viscosity of *solutions* of the same gelatin salts, and that this influence is due, in the case of suspensions, to the influence of the Donnan equilibrium upon the swelling of the particles.

The volume  $V$  of gelatin occupied in 100 cc. of the suspension was determined by filtering and deducting the volume of the filtrate



from the total volume of the suspension. Knowing the viscosity we can calculate Einstein's constant  $c$  according to the formula

$$\left(\frac{\eta}{\eta_0} - 1\right) \frac{100}{V} = c$$

where  $c$  should be 2.5 if  $V$  is sufficiently small.

The values in Table I show that Einstein's formula gives the correct values for viscosity when the volume,  $V$ , of the gelatin is small, since in that case  $c$  is equal, or nearly equal, to 2.5, as his formula demands.

When, however, the volume is larger, the value for  $c$  exceeds 2.5. The fact that the value for  $c$  exceeds 2.5 when the relative volume occupied by the particles in the solution is large, was found also by

TABLE I.

$V$	$\frac{\eta}{\eta_0}$	$c$
<i>ca.</i>		
12	1.292	2.5
17	1.480	2.8
18	1.792	4.4
20.5	2.064	5.1
20.5	2.020	4.9
20	1.855	4.2
18	1.625	3.5
16.5	1.542	3.3

Hatschek,<sup>2</sup> Smoluchowski,<sup>3</sup> and Arrhenius.<sup>4</sup> Hatschek replaced the value 2.5 in Einstein's formula by a larger one, namely 4.5. This, however, meets in our case with the difficulty that the value  $c$  shows a drift reaching a maximum when the volume of the gelatin particles is a maximum. This difficulty is largely avoided in Arrhenius's formula and we have to change from Einstein's formula to that of Arrhenius whenever the relative volume of the particles in solution or suspension exceeds the limits of the applicability of Einstein's formula, as we shall see in the next chapter.

<sup>2</sup> Hatschek, E., *Kolloid Z.*, 1913, xii, 238; 1920, xxvii, 163.

<sup>3</sup> Smoluchowski, M., v., *Kolloid Z.*, 1916, xviii, 190.

<sup>4</sup> Arrhenius, S., *Meddelanden from K. Vetenskapsakademiens Nobelinstitut*, 1917, iii, No. 21.

The experiments on the viscosity of suspensions of powdered gelatin in water have, therefore, led to the result, first, that the influence of pH, of the valency of ions, and of the concentration of neutral salts on the viscosity of *suspensions* of finely powdered gelatin in water is similar to the influence of these three agencies on the viscosity of gelatin *solutions*; second, that the influence of electrolytes on the viscosity of the suspensions is due to the variation of the swelling (or relative volume) of the suspended particles; and third, that this latter fact explains why the Donnan equilibrium determines also the variation of viscosity of these suspensions. If it could be shown that a solution of gelatin contains also some (submicroscopic) particles of solid jelly (capable of swelling), we should understand at once why electrolytes influence the viscosity of gelatin solutions in a similar way as they influence the swelling, osmotic pressure, or the P.D. of these solutions. We have only indirect means of testing this occlusion theory of viscosity in the case of gelatin.

2. If these ideas are correct it would follow that if we melt a suspension of 0.5 gm. of powdered gelatin, in 100 cc. of water the viscosity of the 0.5 per cent solution of gelatin should be considerably lower than the viscosity of the suspension if measured at the same temperature, since in melting, part of the solid particles of the suspension should be transformed into individual molecules or ions or into particles too small to occlude water. In other words, as a consequence of the melting there should be a diminution of the relative volume occupied by the solid gelatin held originally in suspension.

This experiment was tried in the following way: 0.5 gm. of powdered gelatin was put into 100 cc. of water containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12.5, 15, and 20 cc. of 0.1 N HCl to bring the gelatin to different pH. The suspension was allowed to stand 1 hour at 20° to bring about the swelling of the particles and the viscosity of the suspension was measured in a straight viscometer at 20°C. The time of outflow of water through the viscometer at 20° was 48.5 seconds. The upper curve in Fig. 2 gives the ratio of viscosity of suspensions to that of water at 20°C. (When the viscosity is high, the values obtained are a little too great owing to a gravity effect which causes the solid particles to collect above the upper opening of the capillary tube during a part of the time of the experiment, thus increasing

temporarily the density of the suspension.) After the viscosity of a suspension was measured, the suspension was transformed into a solution by heating the suspension to  $45^{\circ}$  for 10 minutes; after that the solution was rapidly cooled to  $20^{\circ}$  and the viscosity of the gelatin solution was now measured with the same viscometer. The lower curve in Fig. 2 shows that the viscosity was now considerably diminished. The abscissæ are the pH of the gelatin solutions.

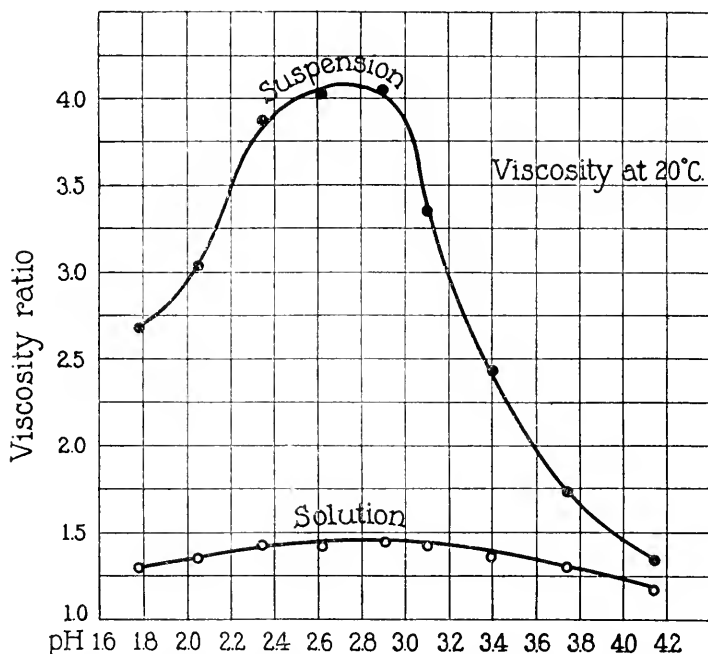


FIG. 2. Difference in the viscosity of a suspension of 0.5 gm. of powdered gelatin in 100 cc. and of the solution of the suspension in the same liquid; both viscosities were measured at  $20^{\circ}\text{C}$ .

Another test of the occlusion theory of viscosity is as follows. When we heat a 1 per cent solution of isoelectric gelatin rapidly to  $45^{\circ}$  and cool it rapidly to a lower temperature, *e.g.*,  $20^{\circ}\text{C}$ ., the solution will ultimately set to a continuous gel but will steadily increase its viscosity before this stage is reached. It is natural to assume that the formation of a continuous jelly is preceded by the formation of smaller masses of jelly, which at first are submicroscopic and later

touch each other, forming a continuous jelly. Hence the longer a solution of isoelectric gelatin stands at 20°C. the greater the number of submicroscopic solid pieces of jelly formed in the solution. The submicroscopic pieces of jelly surrounded by a true solution of isolated molecules of gelatin in water are compelled to regulate the amount of water they occlude by the Donnan equilibrium. Hence when we add some HCl to a 1 per cent solution of isoelectric gelatin after

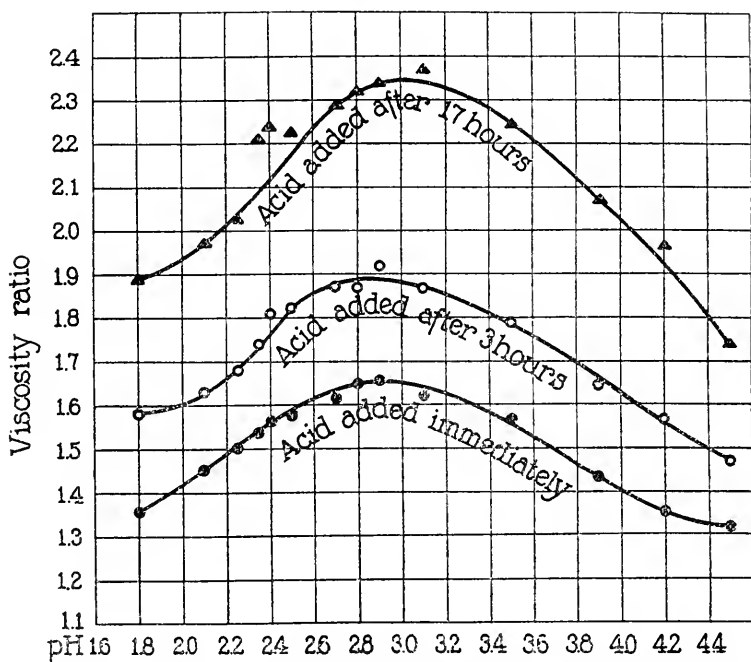


FIG. 3. Increase in viscosity when acid is added to solutions of isoelectric gelatin after they had been standing for 3 and 17 hours respectively.

the solution had been standing for some hours at 20° we should expect to find a higher viscosity than when we add the same amount of acid to the gelatin solution immediately after it had been rapidly heated to 45°C. and rapidly cooled to 20°C.

This experiment turns out as expected, as is shown in Fig. 3. When we bring newly prepared 0.5 per cent solutions of isoelectric gelatin to different pH by the addition of HCl we obtain a viscosity curve like the lowest in Fig. 3. When, however, we let the 0.5 per cent iso-

electric gelatin solution stand for 3 hours at 20°C. before adding the acid, we get a parallel curve but higher than the first one (middle curve, Fig. 3), for the reason that during the 3 hours an additional number of solid jelly particles capable of swelling has been formed. If we let the solution of isoelectric gelatin stand for 17 hours at 20°C., the curve (upper curve, Fig. 3), is still higher though practically parallel with the first curve except at the summit. It is probable that on standing the size of individual particles also increases and the greater the size the greater the viscosity, since the viscosity is chiefly but not exclusively a function of the relative volume of the particles.

These and similar experiments agree with the occlusion theory but not with the hydration theory; since there is no reason why the degree of hydration of gelatin should increase the longer isoelectric gelatin is kept at 20°C.

3. The fact that the viscosity of certain protein solutions is comparatively high, especially in the case of gelatin, has led to the suggestion that proteins might possess a different type of viscosity not present in solutions of crystalloids and that this second type of viscosity might be connected with a certain structure in the protein solution.

“Bearing in mind the possibility that protein solutions may contain a preformed molecular structure analogous to that of the jellies or coagula which they can form, we are strongly impelled towards the belief that the type of viscosity which solutions of proteins exhibit may in some manner owe its existence to this structure, and not to the type of internal friction which hinders molecular and ionic motion. Thus a netlike structure, such as a tennis net, will offer no hindrance to the passage through it of a quickly moving body which is smaller than its meshes, other than that which is due to the fact that the material which composes the net occupies a small fraction of the area which the body must traverse, but to any force which involves deformation of the structure, for instance, a force which seeks to drag it through a small tube, it will offer a very considerable resistance.”<sup>5</sup>

<sup>5</sup> Robertson, T. B., *The physical chemistry of proteins*, New York, London, Bombay, Calcutta, and Madras, 1918, 324–325.

This assumption of a second type of viscosity seems unnecessary since it is possible to account for the viscosities of protein solutions on the basis of Einstein's law when the relative volume occupied by the protein in solution is small, and on the basis of Arrhenius's formula when the volume exceeds the limits within which Einstein's formula holds. According to our view the former is true when the protein exists in the solution exclusively or almost exclusively in the form of isolated molecules or ions or particles too small to occlude water and this seems to be the case for solutions of crystalline egg albumin, at ordinary temperature, at a pH above 1.0 and when the concentration is not excessive.

The viscosity of such protein solutions is not only of a low order of magnitude but is not influenced by electrolytes in the way as is, *e.g.*, their osmotic pressure. Where we have viscosities of a higher order of magnitude, as in the case of gelatin solutions, we notice that the viscosity is influenced by electrolytes in the same way as is the osmotic pressure of these solutions. In the case of such proteins we assume that both the high order of viscosity as well as the characteristic influence of electrolytes on the viscosity are due to the same cause; namely, the existence in the gelatin solution of a certain number of submicroscopic particles of solid jelly occluding large masses of water, the exact amount of which is regulated by the Donnan equilibrium. The occlusion of large masses of water increases the relative volume occupied by the gelatin in solution so that Einstein's formula is no longer applicable. We are dealing, however, in both cases with the same type of viscosity which is primarily a function of the relative volume occupied by the protein particles in the solution.

Measurements of the influence of concentration on the viscosity of solutions of crystalline egg albumin of pH 5.1, *i.e.*, quite near the isoelectric point, were carried out at 15°C. and showed that the viscosity is under these conditions practically a linear function of the concentration (Fig. 4). If Einstein's formula  $\frac{\eta}{\eta_0} = 1 + 2.5 \varphi$  can account for the measurements, it should be possible to calculate the volume  $\varphi$  of the albumin in 100 cc. of solution from

$$\varphi = \left( \frac{\eta}{\eta_0} - 1 \right) \frac{100}{2.5}$$

By dividing the weight of albumin in solution by its volume, we should obtain the density of albumin which should be according to the direct determination not far from 1.36 (Arrhenius).<sup>4</sup> Table II shows that if we calculate the density of solutions of albumin chloride on the basis of Einstein's formula we obtain values which only vary inside the limits of accuracy of the experiments from the density deter-

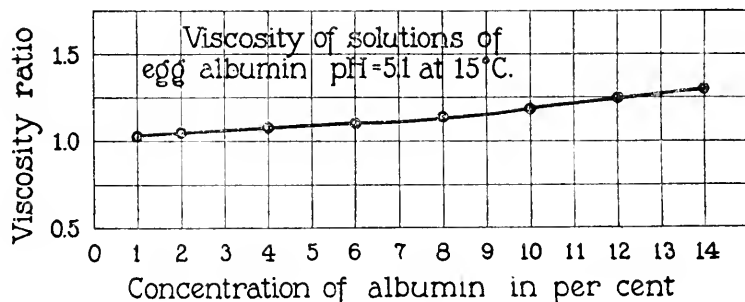


FIG. 4. Viscosity ratio of solutions of crystalline egg albumin near the isoelectric point. Inside the concentrations used, the viscosity ratio is nearly a linear function of the concentration.

TABLE II.

Concentration of crystalline egg albumin.	$\frac{\eta}{\eta_0} - 1$	Calculated volume of albumin.	Calculated density of albumin.
<i>per cent</i>		<i>cc.</i>	
14	0.290	11.6	1.20
12	0.240	9.6	1.25
10	0.185	7.4	1.35
8	0.132	5.3	1.51
6	0.100	4.0	1.50
4	0.074	2.96	1.36
2	0.042	1.7	1.17

mined directly. The time of outflow of water through the viscometer was 227 seconds at 15°C.

Entirely different results should be expected in the case of solutions of gelatin if the high viscosity is due to swollen particles of solid jelly contained in the solution and not due to the existence of a network, as some authors assume. When we plot the curve for the influence of concentration on the viscosity of *solutions of isoelectric gelatin* we get

curves like those of Fig. 5, which are no longer straight lines as in the case of crystalline egg albumin but curved lines. The curvature is the smaller the higher the temperature.

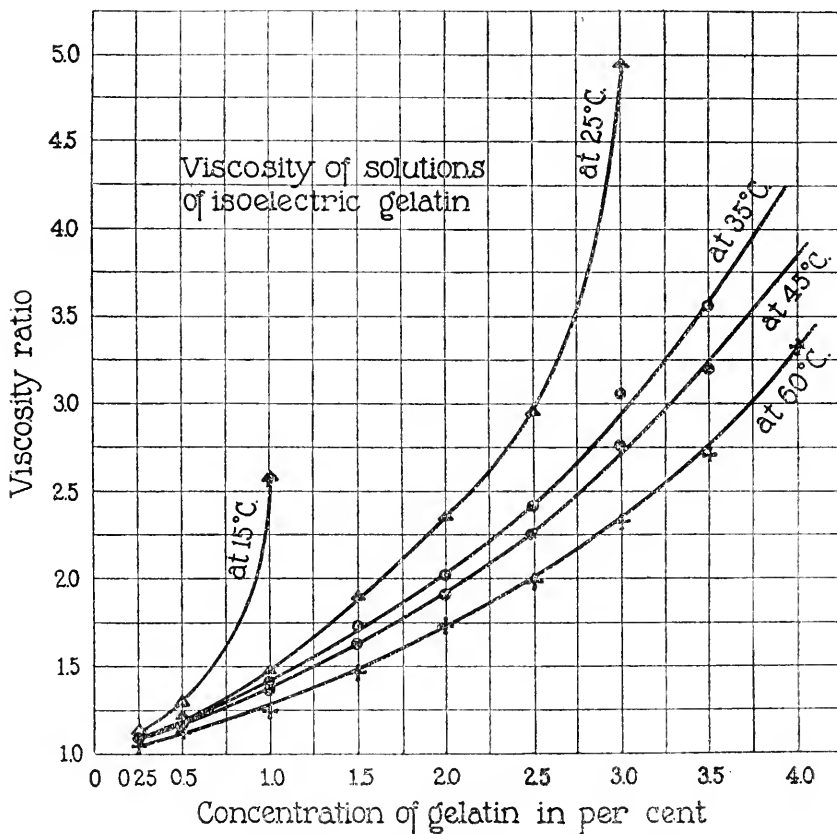


FIG. 5. Influence of concentration on the viscosity of solutions of isoelectric gelatin.

In these experiments the gelatin solution was first heated rapidly to 45° and then brought rapidly to the temperature indicated in the figure. The formula of Einstein can no longer be applied but the formula of Arrhenius gives fair agreement

$$\text{Log } \frac{\eta}{\eta_0} = \vartheta \varphi$$



where  $\varphi$  is the relative volume and  $\vartheta$  is a constant. In other words, if Arrhenius's formula can account for the viscosity of these solutions the logarithm of the viscosity ratio when plotted over the concentration (Fig. 6), should give a straight line. The agreement of the value for 45° and 35° with this theory is satisfactory (considering the limits of accuracy of the measurements), (Fig. 6) the logarithms

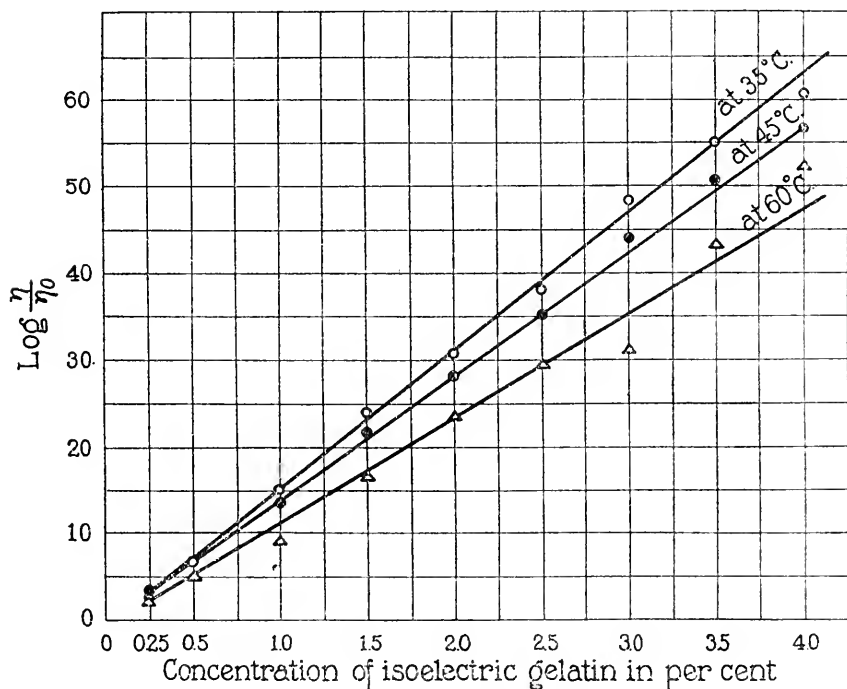


FIG. 6. Showing that the logarithms of the viscosity of solutions of isoelectric gelatin of different concentrations when plotted over the concentration as abscissæ are straight lines.

of the viscosity increasing practically in direct proportion with the concentration (*i.e.*, the relative volume) of the gelatin in the solution (Table III). At 60° the agreement is not quite so good but still recognizable. At 25°, however, it is satisfactory only at the lowest concentrations but at the higher concentrations the viscosity grows more rapidly than the concentration. The reason for this, however, is obvious since at this temperature the gelatin solution solidifies

so rapidly that the viscosity measurements were no longer possible for a concentration of 3.5 per cent gelatin solution, and for this reason the value of the viscosity of a 2 or 3 per cent solution is already too high on account of the mechanical hindrances of the flow of the solution through the viscometer owing to partial solidification.

From these experiments we may therefore draw the conclusion that since the viscosity measurements of gelatin solutions agree fairly well with Arrhenius's viscosity formula the variation of the viscosity of gelatin solutions must be due to variations in the relative volume occupied by the gelatin in solution; and since the gelatin used in these experiments was isoelectric, these variations cannot possibly

TABLE III.

Concentration of solution of iso- electric gelatin.	$\frac{\eta}{\eta_0}$			
	60°C.	45°C.	35°C.	25°C.
<i>per cent</i>				
0.25	0.0236	0.0306	0.0269	0.0374
0.5	0.0504	0.0682	0.0682	0.0792
1.0	0.0930	0.1350	0.1475	0.1685
1.5	0.1656	0.2135	0.2367	0.2765
2.0	0.2350	0.2796	0.3057	0.3701
2.5	0.2953	0.3512	0.3811	0.4691
3.0	0.3094	0.4409	0.4832	0.6941
3.5	0.4321	0.5051	0.5514	Solidifies
4.0	0.5214	0.5660	0.6043	

be ascribed to a hydration of gelatin ions. The only other way by which the isoelectric gelatin particles can increase their volume is through the occlusion of water by submicroscopic particles of solid jelly. Since the amount of water occluded by solid jelly is regulated by the Donnan equilibrium this will explain why the influence of electrolytes on the viscosity of a gelatin *solution* is similar to that of electrolytes on the viscosity of *suspensions* of powdered gelatin in water.

The connection between the tendency of gelatin solutions to set to a jelly and their high viscosity lies therefore in this, that the setting to a jelly is preceded by the formation of submicroscopic solid particles of gelatin capable of swelling and of occluding comparatively large

quantities of water whereby the relative volume occupied by the gelatin in the solution is enormously increased. That this interpretation is correct is proved by experiments on the viscosity of solutions of casein chloride which have no tendency to set to a jelly but which contain, side by side with isolated casein ions and molecules, small particles in suspension, occluding water and capable of swelling.

4. It has already been shown in a preceding paper that the pH influences the viscosity of casein solutions in a similar way as it influences the viscosity of gelatin solutions, and we have convinced ourselves that the addition of neutral salts has a similar depressing effect on the viscosity of casein chloride solutions as it has on the viscosity of solutions of gelatin chloride. Yet casein solutions have no tendency to set to a jelly (as far as the writer has been able to observe) and we cannot therefore attribute the influence of electrolytes on the viscosity of casein chloride solutions to a second type of viscosity. But in the case of casein chloride solutions we can prove directly that the influence of electrolytes on the viscosity has its source in the swelling of casein particles.

The material used in our experiments was a fine dry powder of nearly isoelectric casein prepared after VanSlyke and Baker.<sup>6</sup> Particles of equal size of grain (between mesh 100 and 120) were sifted out and 1 gm. of such powder was put into 100 cc. each of solutions of HCl of different concentration to bring the casein to varying pH.<sup>7</sup> A microscopic examination of the granules showed that they underwent a swelling which was a minimum at the isoelectric point, which increased with increasing hydrogen ion concentration until it reached a maximum and which then diminished again with a further increase in the hydrogen ion concentration. Hence the volume of the casein particles suspended in the HCl varied in a similar way with the pH as the volume of suspended particles of gelatin.

This swelling could also be observed when the suspension was put into 100 cc. graduates and the suspended particles were allowed to settle. The volume of the sediment was a minimum at the isoelectric

<sup>6</sup> Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

<sup>7</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 547.

point increasing with increasing hydrogen ion concentration of the solution and finally decreasing again. But the curves for swelling and for volume of sediment were only parallel at the beginning, since the swelling (which occurred at once) was followed by some of the casein going into solution, or into suspension in the supernatant milky liquid. The longer the experiment lasted the smaller the volume of the sediment became and the larger the mass which went into the supernatant solution. This is expressed in Fig. 7. The upper curve represents the volume of the sediment after 1 hour. The

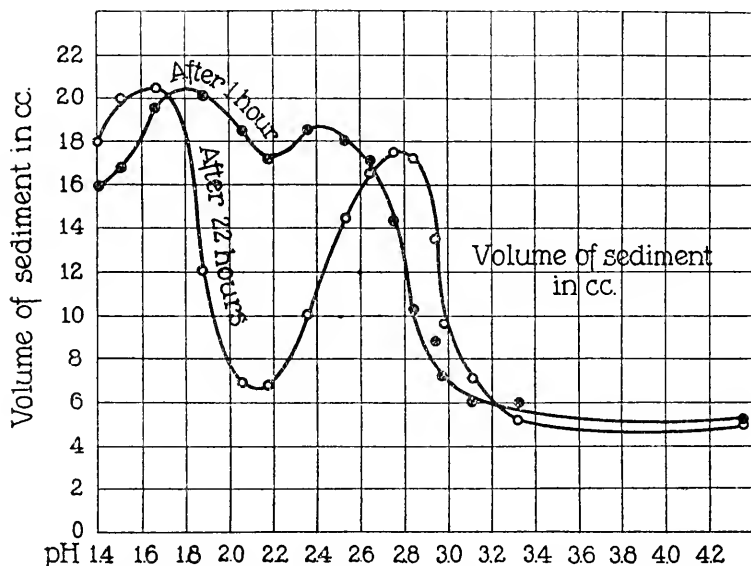


FIG. 7. Swelling and solution of casein chloride in 1 and 22 hours at 20°C.

suspension of 1 gm. of casein in 100 cc. HCl of different concentration had been kept for 1 hour at 20°, had been shaken repeatedly but not frequently and the suspension was then passed into 100 cc. graduates and allowed to settle at 20°C. After 2 hours the volume of the sediment was measured and the volumes are the ordinates of the curve marked "after 1 hour" in Fig. 7. A similar experiment was made in which the suspension of casein was kept for 22 hours at 20°C. and was allowed to settle during 6 hours also at 20°. The volumes are the ordinates of the second curve in Fig. 7 marked "after 22 hours." The abscissæ are the pH of the total solution and suspension.

The curve "after 1 hour" is clear, since it is chiefly the expression of the variation of the degree of swelling of the casein particles since not as much has gone into solution as after 22 hours. We notice that the volume occupied by the solid particles in the 1 hour curve is a minimum at the isoelectric point, that it rises steeply after pH 3.1, and it drops at 2.2, and that a second drop commences at pH 1.8. The two drops have a different cause. The drop at pH 1.8 is due to a diminution of the degree of swelling of the sediment, while the drop at 2.2 in the 1 hour curve is due to the fact that at 2.2 the solubility of casein chloride is a maximum. This conclusion is sup-

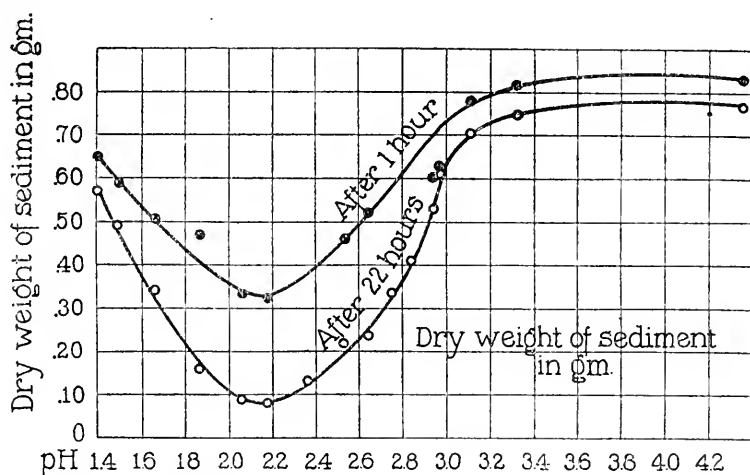


Fig. 8. Dry weight of sediment of casein chloride solutions after 1 and after 22 hours.

ported by the fact that the drop at 2.2 increases in time and is very considerable after 22 hours (Fig. 7), while otherwise the 1 hour and the 22 hour curves show only minor differences.

The proof that this interpretation in the volume curves of Fig. 7 is correct is furnished by Fig. 8 where the ordinates are the dry weights of the sediments, the volumes of which are given in Fig. 7. 1 gm. of powdered casein had when dried for 24 hours at between 90 and 100° a dry weight of 0.87 gm.

That part of the casein chloride which goes into the supernatant liquid (*i.e.*, which is not contained in the sediment) consists of two

constituents, namely, first, solid submicroscopic (and microscopic) particles in suspension which in due time would have settled, and second, isolated casein ions and molecules. The solid particles in the supernatant liquid (unless they are below the limit required to occlude water) undergo the same swelling under the influence of the Donnan equilibrium as the particles of the sediment. In addition, however, we have individual casein ions in solution (the molecules being probably insoluble since isoelectric casein is practically insoluble) but these ions cannot undergo any swelling and hence do not add materially to the volume and the viscosity. As a consequence the more solid particles of casein chloride are dissolved into isolated casein

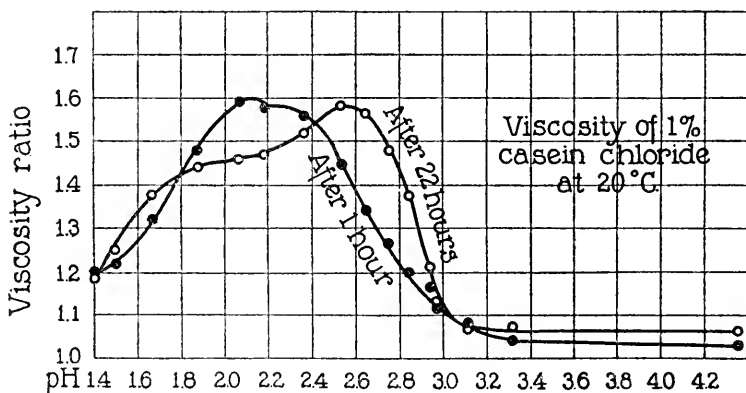


FIG. 9. Viscosity of 1 per cent casein chloride solutions after 1 and 22 hours at 20°C.

ions or particles too small to occlude water the more the relative volume occupied by the casein in the solution should be diminished and this should be accompanied by a diminution in viscosity. If our theory of the origin of the viscosity of the gelatin solutions is correct, it should be possible to prove that where the solubility of the casein chloride solution is a maximum the viscosity curve should show a drop.

The correctness of this inference is supported by the viscosity curves in Fig. 9 which represent the viscosity after 1 hour and after 22 hours. The experiments are the same as those referred to in Figs. 7 and 8. The viscosity of the total suspension and solution

was measured in a straight viscometer with a time of outflow for water of 48.4 seconds at 20°C. The curve for the viscosities after 1 hour is the expression chiefly of the swelling, since casein chloride goes only slowly into solution at 20°C. The curve is almost continuous and has its maximum in the region between pH 2.1 and 2.4, where also the swelling is a maximum. There is, however, a slight depression at pH 2.2 where the solubility of the casein is a maximum.

The curve for the viscosities after 22 hours shows, however, a distinct saddle at pH 2.2 where the solubility of casein chloride is a maximum. This agrees with the assumption that the high viscosity is due to swollen particles of casein, a certain quantity of which had been dissolved at or near pH of 2.2. This solution of the particles capable of swelling beneath that size where they no longer can occlude water must diminish the relative volume of the casein and cause a diminution of the viscosity. Below a pH of 1.8 where the solubility of the casein is considerably diminished, the 1 hour and the 22 hours viscosity curves (Fig. 9) no longer differ materially. As a consequence of the saddle the maximum of the viscosity curve after 22 hours now lies at pH 2.6.

Since the point at issue, namely the diminution of the viscosity when solid submicroscopic particles, capable of swelling, are dissolved into particles so small that they no longer can occlude water is so fundamental for the theories of viscosity and of colloidal behavior in general that it seemed necessary to look for a more striking proof than that given in the experiment quoted. For this purpose measurements were made on 1 per cent casein chloride solutions prepared from very finely powdered casein particles sifted through a 200 mesh. In order to get a more rapid dispersion of the particles the experiment was carried out at 40°C. The time of outflow of water through the viscometer at 40° was 35.5 seconds. Fig. 10 gives the results. The viscosity measurements were made at four different times; namely, first, immediately after the powdered casein was put into the HCl; then after 1, 3, and 6 hours. During this time the casein chloride solutions were kept at 40°C. The viscosity curve taken immediately after the suspensions were prepared is continuous and is the expression of the swelling which occurred in the few minutes which elapsed in the preparation of the suspensions and during

which the casein was at  $40^{\circ}\text{C}$ . The maximum swelling occurred at about pH 2.3. At this time the amount of casein dissolved into separate casein ions was negligible. The curve resembles the 1 hour curve in Fig. 9 except that the depression due to the solution of some casein chloride in Fig. 9 is lacking in Fig. 10. After  $1\frac{1}{2}$  hours the second measurements of viscosity were taken, and the reader will notice from Fig. 10 that the viscosity had dropped considerably in the neighborhood of pH 2.2, where the solubility of casein chloride is the greatest, and the maximum depression is at pH 2.1 where also the solubility is a maximum. With a further lowering of the pH the viscosity rises again. The maximal viscosity in the  $1\frac{1}{2}$  hours

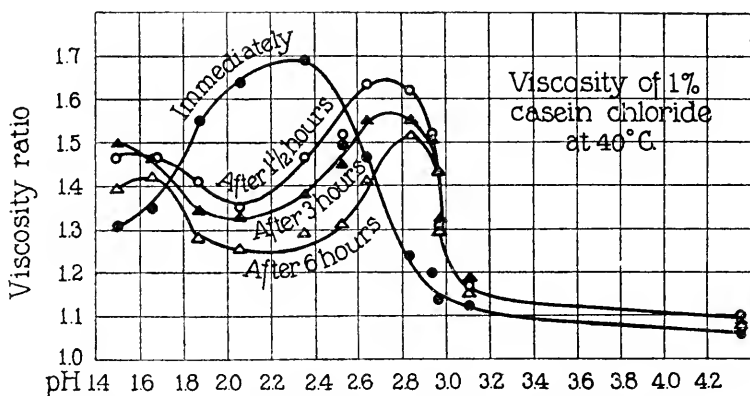


FIG. 10. Diminution of viscosity through solution of solid particles of casein chloride.

series is now at pH of about 2.7 or 2.8 where it was also in 22 hours series in Fig. 9. The later viscosity measurements, after 3 and 6 hours (Fig. 10), confirm these conclusions.

5. These experiments leave little doubt that the high viscosity of protein solutions is due to the existence of particles occluding large quantities of water the amount of which is regulated by the Donnan equilibrium while the isolated ions of proteins in solution or the particles too small to occlude water have no share in the causation of high viscosities.

The quantities of water which can be occluded in a solid jelly of gelatin are enormous. If we assume the molecular weight of gelatin



to be of the order of magnitude of about 12,000 a solid gel of 1 per cent originally isoelectric gelatin contains over 60,000 molecules of water to 1 molecule of gelatin. It is out of the question that such masses of water could be held by the secondary valency forces of the gelatin and water molecules. Swelling casein particles occlude much less water and for this reason the viscosity of casein chloride solutions never becomes as high as that of gelatin solutions, containing equal masses of protein per 100 cc. of solution.

It is well known (and we have made use of the fact earlier in this paper) that the viscosity of a gelatin solution that had been heated to 45°C. and is allowed to stand at 20° increases steadily even if the concentration is not high enough to permit the setting of the solution to a jelly. This increase in viscosity on standing seems to be caused by the gradual increase in the number of solid particles of jelly due to the collision of gelatin molecules or ions, these solid particles occluding water and thus increasing the apparent volume occupied by the gelatin in the solution. The gradual formation of solid particles of jelly from isolated molecules is most rapid at the isoelectric point and is retarded by the addition of acid; while the addition of acid increases the swelling of those particles of jelly which already exist in the solution. It is well to keep in mind that the addition of acid to a solution of isoelectric gelatin has thus two opposite effects on the viscosity of gelatin solutions.

According to Zsigmondy<sup>8</sup> Smoluchowski has explained (in a paper which has not been accessible to the writer) the increase in the viscosity through coagulation of a solution of aluminium oxide by the assumption of an occlusion of liquid between the particles. Smoluchowski calculates from the increase of viscosity during coagulation of aluminium oxide that the coagulating particles occupy a volume 400 to 500 times as great as that occupied by the dry material itself. This apparent increase of volume he explained through the aggregation of needleshaped particles. It would be of interest to test whether or not the volume of these particles and the viscosity of the suspension of aluminium oxide is also controlled by the Donnan equilibrium.

We are therefore led to a conception of the nature of protein solutions which is somewhat different from that current in the literature of

<sup>8</sup> Zsigmondy, R., *Kolloidchemie*, Leipzig, 2nd ed., 1918, 98.

colloid chemistry, which assumes that proteins form no true solutions but suspensions or emulsions. According to our view protein salts form, as a rule, true solutions consisting of isolated protein ions and molecules, which, however, may contain in addition solid fragments occluding comparatively large quantities of water. These latter particles of solid material are responsible for both the comparatively high viscosity of solutions of certain proteins, and for the influence of electrolytes on viscosity.

6. It is of interest to see whether or not Arrhenius's formula can account for the influence of electrolytes on the viscosity of casein suspensions. If this were the case, the curves representing  $\text{Log } \frac{\eta}{\eta_0}$  should run parallel to curves representing the relative volume occupied by the casein in the solution. We get the values of  $\text{Log } \frac{\eta}{\eta_0}$  from our observations of the relative viscosity which give us  $\frac{\eta}{\eta_0}$ , and we can calculate the volume from the measured volume of the sediment plus the calculated volume of the casein in the supernatant liquid. The latter value we obtain by deducting the dry weight of the sediment from the (known) dry weight of the whole mass of casein put into the water (1 gm. powdered casein, dry weight = 0.87 gm.), and assuming that the casein in the supernatant liquid consists exclusively of suspended particles. This is approximately correct for a 1 hour experiment at 20°. The ordinates in Fig. 11 represent the values for volume thus corrected and the values for  $\log \frac{\eta}{\eta_0}$  while the abscissæ are the pH of the suspensions. The two curves are almost parallel.

It should be stated that these corrected volumes of casein include a certain amount of water between the granules. We are, however, in this case not concerned with the absolute but the relative volume occupied by the casein.

When we add NaCl in different concentrations to a casein chloride solution we notice that the viscosity is diminished as it is in the case of solutions of gelatin chloride. R. F. Loeb found by microscopic observation that this diminution of viscosity was accompanied by a diminution in the degree of swelling of the individual particles of casein which ran parallel to the depression of the viscosity.

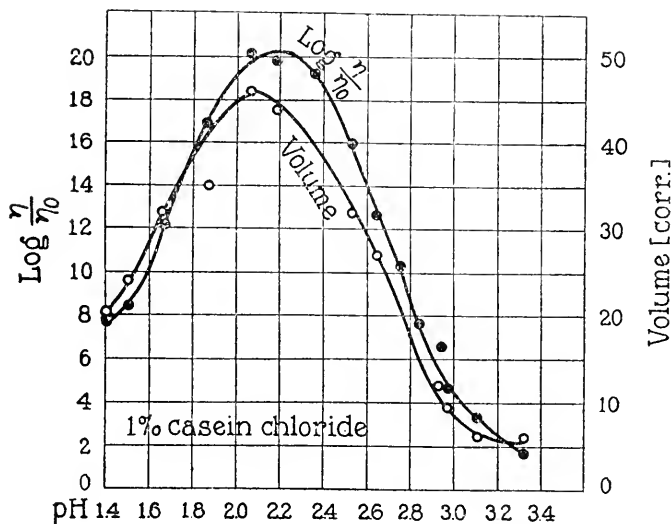


FIG. 11. Similarity of curve for  $\log \frac{\eta}{\eta_0}$  and of relative volume of casein chloride solutions.

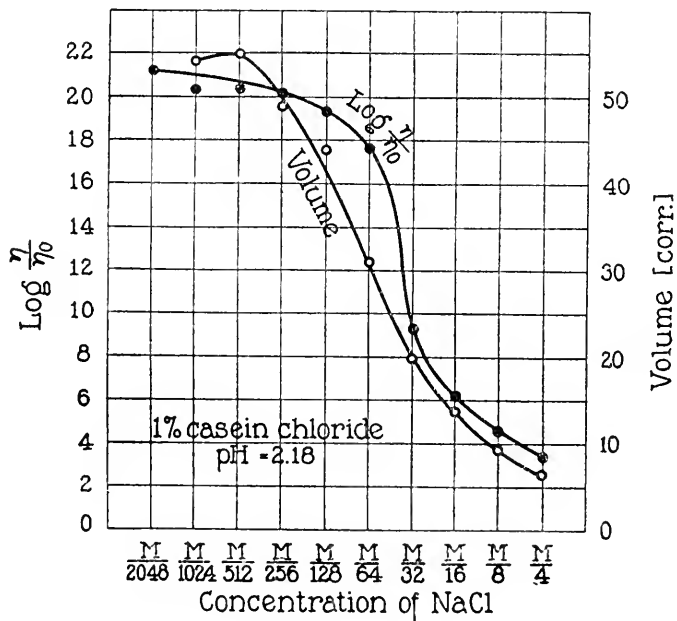


FIG. 12. Similarity of curve for  $\log \frac{\eta}{\eta_0}$  and of relative volume of casein chloride solutions.

1 gm. of powdered casein was put into 100 cc. of  $H_2O$  containing 12.5 cc. of 0.1 HCl and NaCl in concentrations varying from 0 to  $M_4$ . The mixture was shaken occasionally and kept for 16 hours at  $20^\circ$ . Then the viscosity, volume of sediment (after settling for 24 hours), dry weight of sediment (after deduction of the free NaCl contained in the sediment) were determined. When the corrected volume and the values for  $\log \frac{\eta}{\eta_0}$  are plotted as ordinates over the concentration of abscissæ it is found that the two curves agree fairly well (Fig. 12) except where no or little salt was added and where therefore some casein particles had been completely dissolved. In this solution the calculated volume was too high and our curves express the fact. From these experiments we may conclude that the influence of electrolytes on the viscosity of casein solutions or suspensions is due to the swelling of particles of casein suspended in the solution of casein and that the volume of these particles is regulated by the Donnan equilibrium.

#### SUMMARY.

1. The proof is completed that the influence of electrolytes on the viscosity of suspensions of powdered particles of gelatin in water is similar to the influence of electrolytes on the viscosity of solutions of gelatin in water.

2. It has been suggested that the high viscosity of proteins is due to the existence of a different type of viscosity from that existing in crystalloids. It is shown that such an assumption is unnecessary and that the high viscosity of solutions of isoelectric gelatin can be accounted for quantitatively on the assumption that the relative volume of the gelatin in solution is comparatively high.

3. Since isoelectric gelatin is not ionized, the large volume cannot be due to a hydration of gelatin ions. It is suggested that this high volume of gelatin solutions is caused by the existence in the gelatin solution of submicroscopic pieces of solid gelatin occluding water, the relative quantity of which is regulated by the Donnan equilibrium. This would also explain why the influence of electrolytes on the viscosity of gelatin solutions is similar to the influence of electrolytes on the viscosity of suspensions of particles of gelatin.

4. This idea is supported by experiments on solutions and suspensions of casein chloride in which it is shown that their viscosity is chiefly due to the swelling of solid particles of casein, occluding quantities of water regulated by the Donnan equilibrium; and that the breaking up of these solid particles into smaller particles, no longer capable of swelling, diminishes the viscosity.

5. This leads to the idea that proteins form true solutions in water which in certain cases, however, contain, side by side with isolated ions and molecules, submicroscopic solid particles capable of occluding water whereby the relative volume and the viscosity of the solution is considerably increased. This accounts not only for the high order of magnitude of the viscosity of such protein solutions but also for the fact that the viscosity is influenced by electrolytes in a similar way as is the swelling of protein particles.

6. We therefore reach the conclusion that there are two sources for the viscosity of protein solutions; one due to the isolated protein ions and molecules, and the other to the submicroscopic solid particles contained in the solution. The viscosity due to the isolated molecules and ions of proteins we will call the general viscosity since it is of a similar low order of magnitude as that of crystalloids in solution; while the high viscosity due to the submicroscopic solid protein particles capable of occluding water and of swelling we will call the special viscosity of protein solutions. Under ordinary conditions of hydrogen ion concentration and temperature (and in not too high a concentration of the protein in solution) the general viscosity due to isolated ions and molecules prevails in solutions of crystalline egg albumin and in solutions of metal caseinates (where the metal is monovalent) while under the same conditions the second type of viscosity prevails in solutions of gelatin and in solutions of acid-salts of casein; and also in solutions of crystalline egg albumin at a pH below 1.0 and at higher temperatures. The special viscosity is higher in solutions of gelatin than of casein salts for the probable reason that the amount of water occluded by the submicroscopic solid gel particles in a gelatin solution is, as a rule, considerably higher than that occluded by the corresponding particles of casein.



# THE RECIPROCAL RELATION BETWEEN THE OSMOTIC PRESSURE AND THE VISCOSITY OF GELATIN SOLUTIONS.

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1. If we put a suspension of 1 gm. of finely powdered gelatin in 100 cc. of HCl, so that the gelatin has a pH of 3.4, into a collodion bag and dip the latter into a pure solution of HCl (without gelatin) of pH 3.0, the liquid will practically not rise in the manometer connected with the liquid in the collodion bag (except to that slight extent which may be caused by some of the solid gelatin going into solution); in other words, we get no osmotic pressure. The reason is as follows: in this experiment the Donnan equilibrium is established between each individual granule of solid gelatin and the solution of HCl inside the bag and since the latter diffuses freely through the membrane there is in this case no difference of pH inside and outside the collodion membrane, and hence no difference of osmotic pressure on the opposite sides of the membrane (unless some of the solid gelatin goes into solution).

If we make this experiment, however, with a 1 per cent *solution* of gelatin chloride of a pH of 3.4 inside the collodion bag, while on the other side of the membrane we have a HCl solution of pH 3.0 without gelatin, we notice a rapid rise of the column of water in the manometer tube inserted into the collodion bag and the osmotic pressure of the 1 per cent solution of gelatin will be about 450 mm. H<sub>2</sub>O or a little more when equilibrium is established.<sup>1</sup> This proves that the osmotic pressure of a gelatin solution cannot be due to (submicroscopic) solid particles of a jelly, but must be due to particles causing a Donnan equilibrium *across the collodion membrane*. Such particles can only be the individual gelatin ions, since even the smallest

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 691.

particle of gel must cause a Donnan equilibrium to be established between the solid particles and the solution inside the bag.

If we assume that certain protein solutions contain two different constituents, namely, first, isolated ions and molecules, and second, isolated submicroscopic particles capable of occluding water, these two constituents of such solutions must play a different rôle in osmotic pressure and in viscosity. The characteristic influence of electrolytes on the osmotic pressure of protein solutions must be due to the isolated protein ions since these alone can bring about the Donnan equilibrium across the membrane; while the similar influence of electrolytes on the viscosity of protein solutions must be due to the solid submicroscopic particles since these alone are capable of swelling and of giving rise to a Donnan equilibrium between each individual particle and solution.<sup>2</sup> If this inference is correct, it should be possible to demonstrate the existence of a reciprocal relation between the viscosity and the osmotic pressure of those protein solutions which contain isolated protein ions as well as solid protein particles capable of occluding water; *e.g.*, gelatin or casein solutions. It is the purpose of this paper to show that this reciprocal relation between the osmotic pressure and viscosity of such protein solutions actually exists.

Fig. 1 shows that the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin diminishes the more, the more we replace the dissolved gelatin by small granules of powdered gelatin. The ordinates of the upper curve represent the values of the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin at different pH, the pH serving as abscissæ of the curves. The acid used was HCl, and the curve is the usual one given repeatedly in the writer's preceding publications. At the beginning of the experiment the gelatin solution was rapidly heated to a temperature of 45°C. and rapidly cooled to 20°C. and then kept at that temperature throughout the entire experiment. The pH is that of the gelatin solution at the end of the experiment.

The middle curve represents an experiment in which 0.5 gm. of the isoelectric gelatin in solution was replaced by 0.5 gm. of isoelectric powdered gelatin. The latter did not contribute to the osmotic

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 73.



pressure, the observed osmotic pressure being due to the isolated ions of the 0.5. per cent gelatin in solution which determine the Donnan effect, and in addition, to the gas pressure of the isolated gelatin ions and the isolated gelatin molecules. Theoretically, of course, the

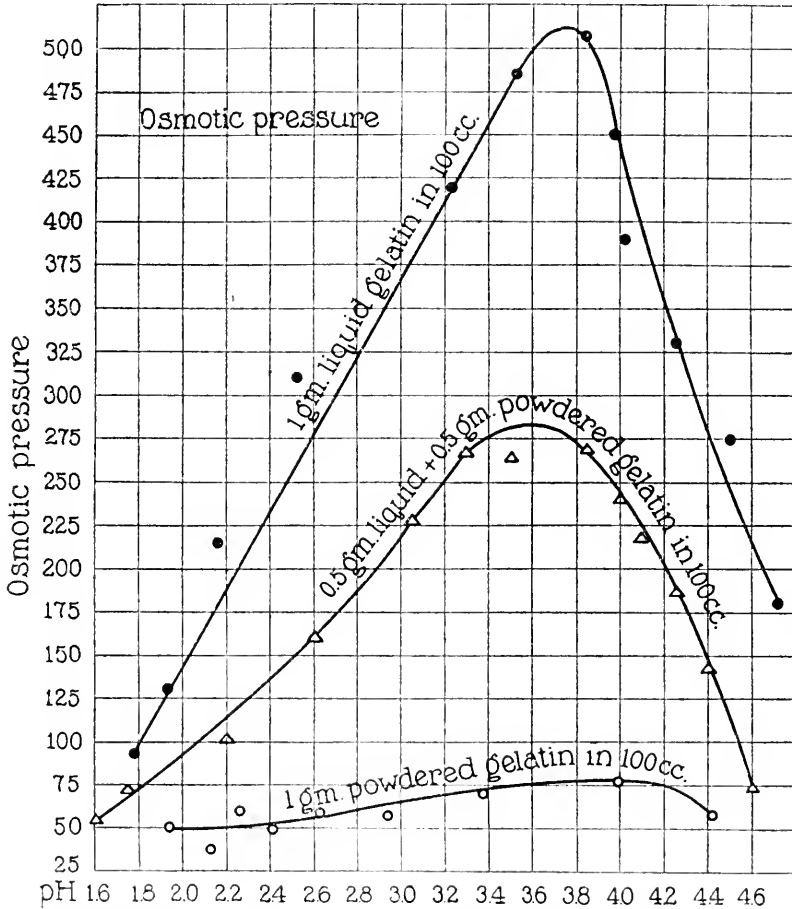


FIG. 1. A suspension of 1 gm. of a fine powder of gelatin in 100 cc. of water has practically no osmotic pressure (lowest curve), while a solution of 1 gm. of the same gelatin has a maximal osmotic pressure of over 500 mm. (uppermost curve). A mixture of 0.5 gm. of powdered and 0.5 gm. of liquid gelatin in 100 cc. water has practically the osmotic pressure of the 0.5 per cent liquid gelatin in 100 cc. of water (middle curve).

coarse particles of gelatin also participate in the osmotic pressure but this effect is negligible on account of the small number of such particles. (The gelatin particles used were of grain size slightly above one sixtieth of an inch diameter.) At the beginning of the experiment the 0.5 per cent solution of gelatin was rapidly heated to 45°C. and rapidly cooled to 20°C., and then the powdered gelatin was added. The pH is that of the 0.5 per cent gelatin solution at the end of the experiment.

The lowest curve represents the osmotic pressure of 1 gm. of powdered isoelectric particles in 100 cc. of HCl of different pH. The slight osmotic pressure observed is probably due to the fact that a little of the gelatin went gradually into solution. This apparently happened to a less extent in a repetition of this experiment and the osmotic pressures observed were still lower than in the lowest curve in Fig. 1. All these osmotic pressure experiments were made in a thermostat at 20°C.

The viscosity is affected in exactly the opposite way from the osmotic pressure if part of the dissolved gelatin is replaced by solid particles of gelatin. The more dissolved gelatin is replaced by solid particles of gelatin the higher the viscosity, a result to be expected from the experiment and conclusions published in the preceding paper.<sup>2</sup>

Solutions of 0.5, 0.625, 0.750, 0.875, and 1.0 gm. of isoelectric gelatin were heated quickly to 45°C. and cooled quickly to 20°C. and so much powdered gelatin of pH 7.0 was added as to bring the total gelatin in 100 cc. to 1 gm.; *i.e.*, to a 0.5 per cent solution of gelatin was added 0.5 gm. of powdered gelatin (between mesh sizes 100 and 120), and to a 0.875 per cent solution of liquid gelatin was added 0.125 gm. of powdered gelatin, while no powdered gelatin was added to the 1 per cent solution of liquid gelatin. The different mixtures were brought to different pH through the addition of different quantities of HCl and the solutions were allowed to stand for 1 hour before the viscosities were measured in order to give the powdered gelatin a chance to swell. The results of the measurements are represented in Fig. 2. The reader will see that within the range of the pH between 3.6 and 1.4 the viscosity is the greater, the more liquid gelatin is replaced by powdered gelatin. This supports the idea that the

influence of electrolytes on the viscosity of gelatin solutions is due to the influence of the electrolytes on the swelling of solid submicroscopic particles of gel in the solution.

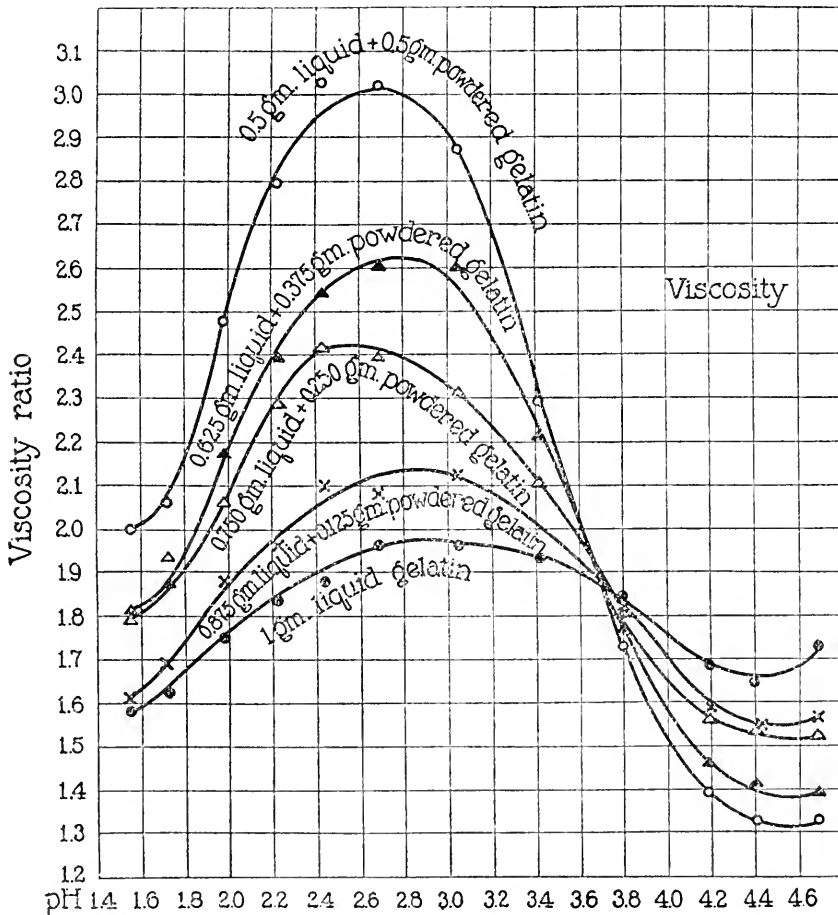


FIG. 2. The influence of replacing liquid by powdered gelatin on viscosity is exactly the reverse as on osmotic pressure. The more the powdered particles replace the liquid gelatin the higher the specific viscosity.

The nature of the curves in Fig. 2 between pH 4.6 and 3.8 requires an explanation. The curves are here the lower the more liquid gelatin is replaced by solid gelatin. This is due to the fact that it was neces-

sary to let the suspensions stand for at least 1 hour to allow the particles of powdered gelatin to swell before the viscosity measurements were made. During this time the liquid gelatin at, or near, the isoelectric point increases rapidly in viscosity while this increase in viscosity is suppressed where the hydrogen ion concentration is higher. This is proved by Fig. 3 which gives the viscosity of the supernatant solutions of gelatin (without the suspended particles) which had been standing for 1 hour. Inside the range of pH 4.4 and 4.6 the viscosity had risen more rapidly on standing than at the lower pH;

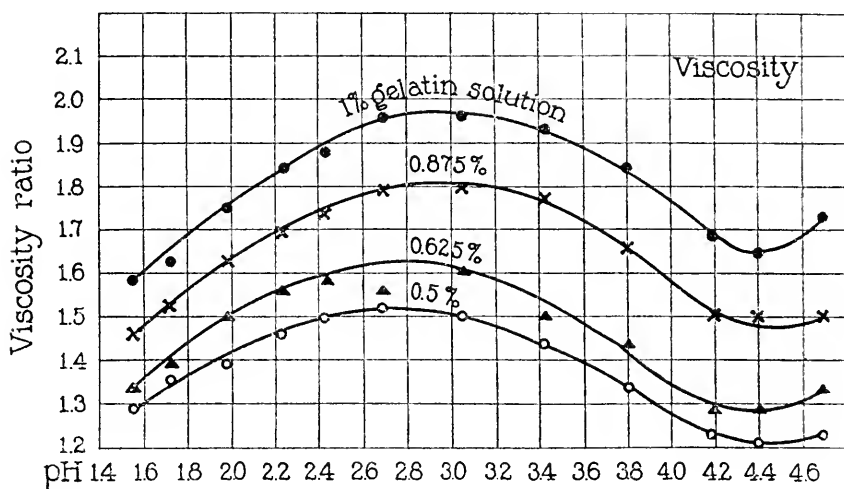


FIG. 3. Increase of viscosity of gelatin solution at the isoelectric point on standing due to the formation of submicroscopic particles of gel.

which means that at, or near, the isoelectric point new submicroscopic particles of solid jelly are constantly formed from the molecules while this process is the slower the higher the hydrogen ion concentration. While thus the addition of acid to a solution of isoelectric gelatin retards the rate of formation of new submicroscopic particles of jelly, it increases the swelling of those already present when the acid is added. On the other hand, powdered particles of isoelectric gelatin in water of pH 4.7 do not increase their volume on standing.

The fact that the addition of acid to a solution of isoelectric gelatin inhibits or retards the formation of new solid particles on standing

is strikingly illustrated by Fig. 4. Gelatin chloride solutions all containing 1 gm. of originally isoelectric gelatin were brought to various pH by adding HCl and were at the beginning of the experiment all heated rapidly to 45° and cooled at once to 20°C. Then the viscosity was measured immediately at 20°. The result is given in the lowest curve in Fig. 4. The solutions were kept at 20° for 1 hour and the viscosity measurements were repeated. The middle curve in Fig. 4

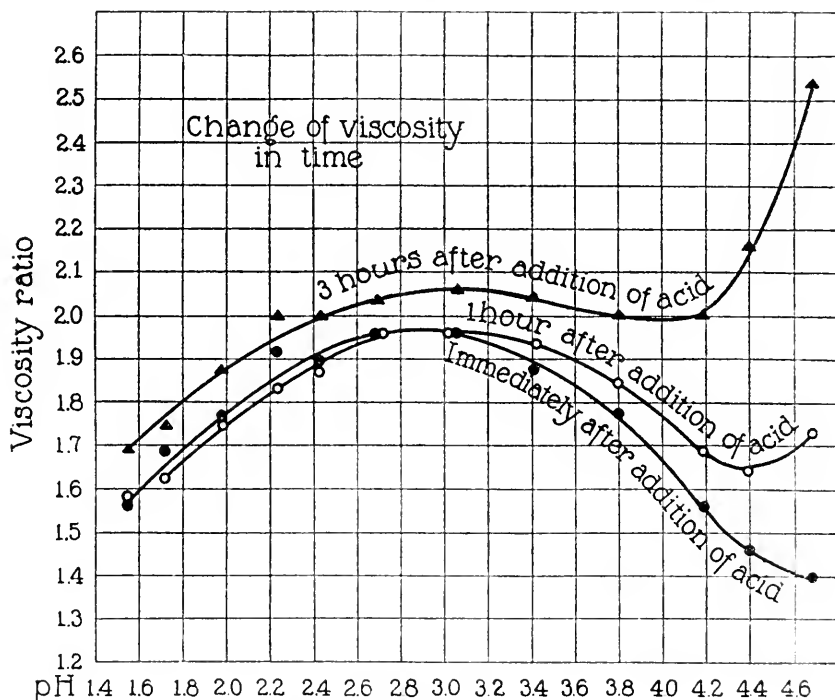


FIG. 4. See legend of Fig. 3.

shows that at this time the viscosity of the isoelectric solution had increased considerably, that of the solutions of lower pH down to 3.0 had increased the less, the lower the pH. Below pH 3.0 no increase in the viscosity had occurred. 2 hours later another viscosity measurement was made; the results are represented in the upper curve of Fig. 4. At the isoelectric point the viscosity had increased enormously, but less and less at lower pH. During all the time and also

during the viscosity measurements the temperature of the solutions was 20°C.

A more exact demonstration of the inhibiting influence of acid on the increase of viscosity of gelatin solutions on standing is given in

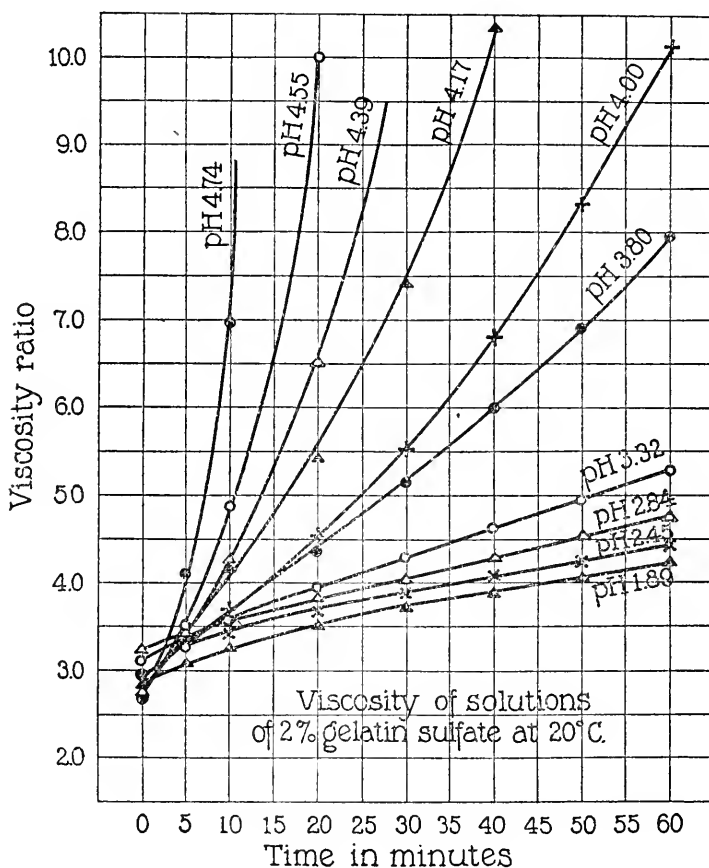


FIG. 5. Increase of viscosity of gelatin sulfate solution of different pH on standing. The increase is most rapid at the isoelectric point, thus proving that the acid retards or prevents the formation of submicroscopic solid particles of jelly on standing.

Fig. 5. Gelatin sulfate solutions containing 2 gm. of originally isoelectric gelatin in 100 cc. were prepared and heated rapidly to 45°C. and cooled rapidly to 20°C., and then kept constantly at 20°C.

Viscosity measurements were made every 5 or 10 minutes. Fig. 5 gives the result. The lower the pH the less the viscosity increases on standing.

There exist, therefore two different and to some extent antagonistic effects of acid on the viscosity of gelatin solution. One effect is due to the swelling of the submicroscopic particles of solid gelatin already present in the solution at the time the acid is added. This effect is regulated by the Donnan equilibrium and increases when (not too much) acid is added to the isoelectric gelatin solution. The second effect consists in retarding the formation of solid particles of jelly on standing. This inhibitory effect of the acid seems to increase steadily with the hydrogen ion concentration of the solution without going through a maximum at a pH above 1.8.

If we now return to the discussion of the curves in Fig. 2 we may say that the results in that part of the curves which belongs to the abscissæ of pH above 3.8 is the expression of the fact that that part of the viscosity which is due to the gelatin in solution had undergone an increase during the hour the solution had been standing at 20° after having been heated to 45°C.; and that the increase caused in the viscosity of the liquid gelatin was a maximum at the isoelectric point, being almost zero at a pH below 3.4; while the addition of acid had the opposite effect on the solid granules of gelatin, since their volume was increased according to the rules of the Donnan equilibrium.

It is necessary that we convince ourselves that a Donnan equilibrium exists when particles of solid gelatin are suspended in a solution of gelatin. That this is actually true was shown in the following way. 0.5 gm. of powdered gelatin was added to 100 cc. of a 0.5 per cent gelatin chloride solution of different pH. The different beakers containing these mixtures were kept for 3½ hours at 20°C. The mass was then filtered through cotton wool and the pH of the filtrate (0.5 per cent gelatin solution) and of the solid gelatin granules were determined, that of the latter after they had been melted. It was found that the pH of the gelatin granules was higher than that of the solution and that the difference followed the Donnan equilibrium equation (Table I), though the result was slightly irregular owing to the fact that it is impossible to free the suspended particles of gelatin completely from the supernatant liquid. When we separate

TABLE I.

pH of gelatin in suspension....	5.12	4.60	4.49	4.18	4.07	3.73	3.45	2.93	2.68	2.34	2.09	1.86	1.77	1.53
pH of gelatin in solution.....	4.98	4.35	4.12	3.91	3.69	3.50	3.14	2.78	2.50	2.28	1.97	1.86	1.72	1.57

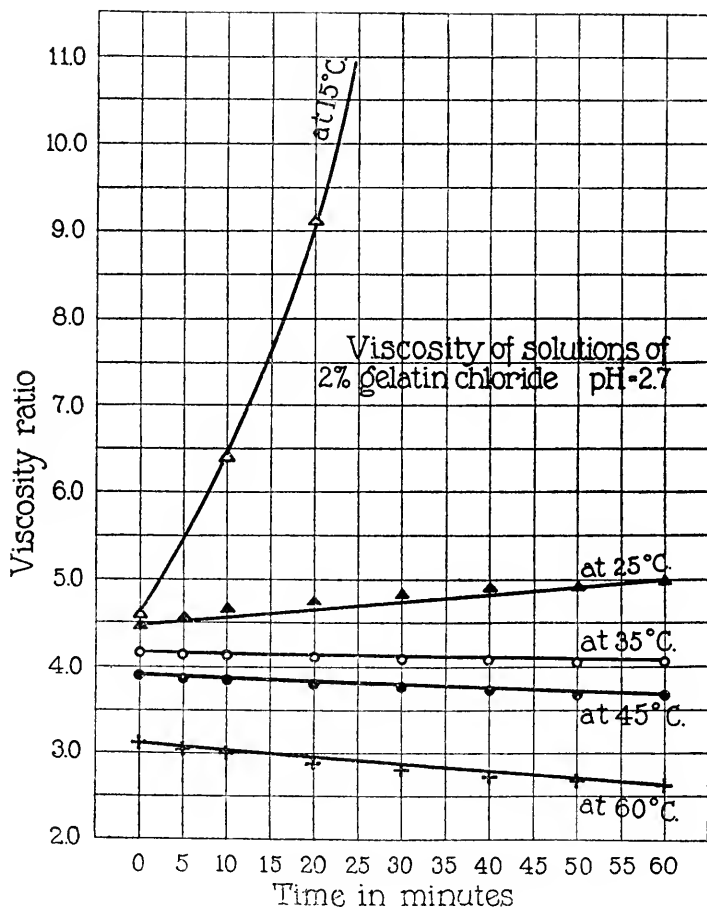


FIG. 6. Influence of temperature on the variation of viscosity of gelatin solutions on standing. Below 35°C. the viscosity of a 2 per cent gelatin chloride solution of pH 2.7 no longer increases but diminishes on standing.



a gelatin solution from water by a collodion membrane we get two equilibria, one across the membrane caused by the protein ions on one side of the membrane; and a second one inside the protein solution caused by the solid particles of jelly.

2. These experiments in which liquid gelatin was replaced by solid particles of gelatin support the idea of a reciprocal relation between the osmotic pressure and the viscosity of gelatin *solutions*. It is, however, possible to give a more direct proof. It was noticed that the viscosity of solutions of gelatin chloride does not always increase on standing but that it diminishes when the temperature exceeds a certain limit. This is shown for a 2 per cent solution of gelatin chloride of pH 2.7 in Fig. 6. The viscosity of such a solution increases on standing very rapidly at 15°C., much less rapidly at 25°C., but diminishes when kept at a temperature above 35°C., and the more rapidly the higher the temperature. This we assume to be due to the fact that at a temperature above 35° the rate of melting of submicroscopic particles of solid jelly exceeds the rate of their formation from isolated ions or molecules.

Several liters of a 0.55 per cent solution of isoelectric gelatin were kept at about 10°C. for 48 hours and at 20° for the next 24 hours. Then the stock solution was divided into two parts. The one part was divided into parts of 90 cc. each, and each part was brought to different pH by adding 10 cc. containing different quantities of HCl. In this way the concentration of originally isoelectric gelatin was, therefore, in every case 0.5 per cent. The second portion was treated in the same way except that before adding the acid the gelatin was kept for 1 hour at 45°C. This was done to melt part of the submicroscopic pieces of jelly assumed to exist in the solution and thus to increase the concentration of the isolated ions and molecules and to diminish the relative quantity of solid submicroscopic particles capable of occluding water and thereby causing the high viscosity characteristic of gelatin solutions. After this second portion of the stock solution of isoelectric gelatin had been kept for 1 hour at 45°C. it was rapidly cooled to 20°, the HCl was added in the way described for the first portion and the solutions were put into collodion bags to measure the osmotic pressure. Each collodion bag contained about 50 cc. of gelatin solution. The temperature now remained constant

at 20°C. for both sets of experiments. It was noticeable from the first that the osmotic pressure of the gelatin solution which had been kept for 1 hour at 45° and which was therefore supposed to have melted into smaller particles was higher than that of the gelatin solution not previously heated. Fig. 7 shows the result after 22 hours. The maximum osmotic pressure was, for the gelatin solution that had been previously heated, 200 mm.  $\text{H}_2\text{O}$ , while it was only 170 mm. for the other gelatin solution not previously heated to 45°C.

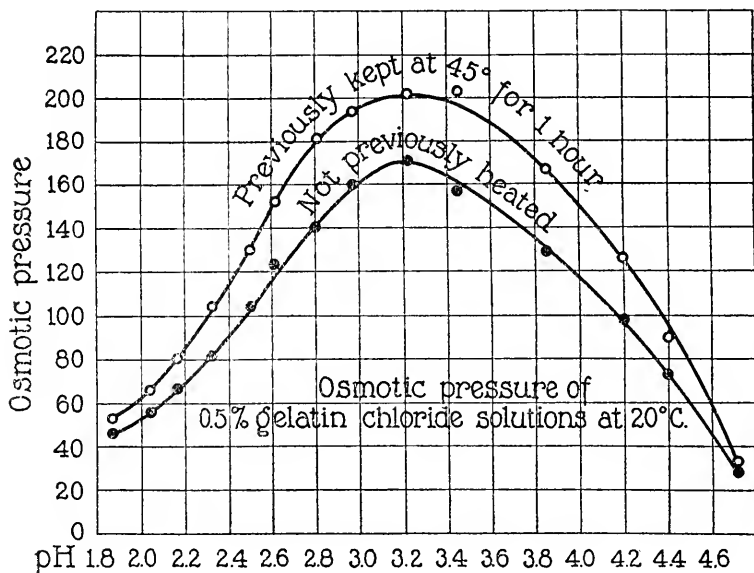


FIG. 7. Showing that the osmotic pressure of a solution of gelatin chloride which has been previously heated to 45°C. for 1 hour and then rapidly cooled to 20°C. is higher than the osmotic pressure of the same solution of gelatin chloride not previously heated.

Then the viscosities were determined at 20° and they gave the opposite result (Fig. 8), the viscosities being considerably higher in the solutions not previously heated to 45° than in the solutions previously heated. This experiment then confirms our expectation that there exists a reciprocal relation between the viscosity of protein solutions and their osmotic pressure, inasmuch as a transformation of solid submicroscopic particles of jelly into isolated protein ions

and molecules diminishes the viscosity but increases the osmotic pressure of the gelatin solution.

As far as the quantitative relations are concerned, the difference in viscosity (Fig. 8) is more striking than the difference in osmotic pressure (Fig. 7). The osmotic phenomena are a more complicated function of the change caused by the transformation of larger aggregates into simple ions, inasmuch as there exists a Donnan equilibrium not only between the gelatin solution and the outside watery solution

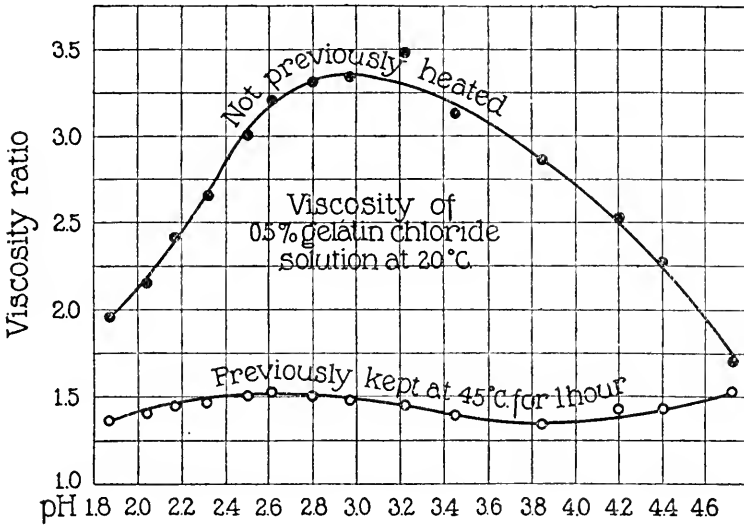


FIG. 8. Showing that the influence of previous heating on the viscosity of 0.5 per cent solutions of gelatin chloride is the reverse of the influence of the previous heating on osmotic pressure (Fig. 7).

but also between each solid particle of gelatin and the true gelatin solution surrounding it.

It was expected that when we put a 1 per cent solution of gelatin of, e.g., pH 3.5, which had been kept for 1 hour at 45° and cooled to 20° into a 1 per cent solution of an identical gelatin chloride solution of pH 3.5, which had not been heated to 45° before being brought to 20°C., water would diffuse from the latter into the former solution. This experiment was carried out with a positive result.

These experiments support the idea expressed in the preceding paper that protein solutions are true solutions which may or may not contain solid particles of protein capable of swelling. In the case of gelatin solutions the formation of submicroscopic particles of solid jelly from isolated molecules or ions is a reversible process and this leads in this case to a reciprocal variation of osmotic pressure and viscosity of such solutions.

This probably explains a phenomenon which has puzzled the writer for a long time; namely, that the osmotic pressures of gelatin solutions of the same pH and concentration of originally isoelectric gelatin occasionally showed variations for which he could not account. It now becomes probable that this was due to a factor which was not taken into consideration, namely, that on standing at room temperature a gradual transformation of isolated molecules or ions into larger aggregates takes place, which must diminish the osmotic pressure but increase the viscosity. This source of variation was eliminated in the viscosity experiments in which the gelatin solution was always heated first to 45°C. and then as soon as this temperature was reached the solution was cooled to the temperature desired for the viscosity measurements. It is probable that the same uniformity of treatment is also required for the osmotic pressure experiments.

This reciprocal relation between osmotic pressure and viscosity exists probably also in the case of solutions of casein salts. Solutions of Na caseinate are less opaque than those of casein chloride (of the same concentration of originally isoelectric casein) which indicates that the Na caseinate solution contains more isolated casein ions and molecules and less submicroscopic solid particles than the solution of casein chloride.

The writer had already shown in a preceding paper that the maximal viscosity of a 1 per cent solution of casein chloride is higher than the viscosity of solutions of Na caseinate of equal concentration of originally isoelectric casein, while the osmotic pressures of solutions of the two salts show exactly the reverse relation, the maximal osmotic pressure of a 1 per cent solution of Na caseinate being almost 700 mm. H<sub>2</sub>O while the maximal osmotic pressure of a 1 per cent solution of casein chloride is only about 200 mm.

The solutions of crystalline egg albumin seem to consist (at ordinary temperature and at not too high a concentration of albumin and of the hydrogen ions) exclusively or almost exclusively of isolated molecules or ions. Since the latter cannot diffuse through a collodion membrane they give rise to a Donnan equilibrium across the membrane and hence only the osmotic pressure of solutions of salts of crystalline egg albumin is influenced by electrolytes in the way demanded, while the viscosity shows such an influence only to a negligible degree.

3. Since in connection with viscosity we have assumed the existence of submicroscopic solid particles of jelly in gelatin solutions we must point out where our ideas agree and where they disagree with the speculations on the rôle of the degree of dispersion current in colloid chemistry. On the basis of these latter speculations it would be assumed that the osmotic pressure of a protein solution is determined directly by the concentration of the protein in solution according to van't Hoff's law. We assume the correctness of van't Hoff's law but the osmotic pressure is only in part determined by the gas pressure of the protein particles, the main source of the osmotic pressure being the unequal distribution of the crystalloidal ions on the opposite sides of the membrane due to the Donnan equilibrium. This has been overlooked by the believers in the dispersion theory. We agree with the believers in the dispersion theory that the melting down of larger aggregates of gelatin ions into isolated gelatin ions must raise the osmotic pressure but disagree in the explanation of this effect; since in our opinion the increase in the number of isolated protein ions must lead to an increase in the distribution of crystalloidal ions on the opposite sides of the membrane according to the Donnan equilibrium and this is the main reason why the osmotic pressure of a gelatin solution is increased when the solid submicroscopic particles of jelly are transformed into isolated ions.<sup>3</sup>

<sup>3</sup> If we wish to measure the true osmotic pressure of a protein solution, free from the complication of the Donnan equilibrium, we must do so at the isoelectric point of the protein. This condition was approximately fulfilled in the experiments of Sørensen (Sørensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915-17, xii, 262) on solutions of crystalline egg albumin, and of Hüfner and Gansser (Hüfner, G., and Gansser, E., *Arch. f. Physiol.*, 1907, 209) on solutions of hemoglobin.

On the other hand, the high viscosity of gelatin solutions, *e.g.*, that part of the viscosity which is due to the volume occupied by the solid particles of jelly, is entirely regulated by the Donnan equilibrium. The dispersion theory was not able to account for the influence of electrolytes on the viscosity and osmotic pressure of protein solutions while this influence is accounted for mathematically by the Donnan theory.

#### SUMMARY AND CONCLUSIONS.

1. These experiments confirm the conclusion that protein solutions are true solutions consisting of isolated ions and molecules, and that these solutions may or may not contain in addition solid submicroscopic particles capable of occluding water.

2. The typical influence of electrolytes on the osmotic pressure of protein solutions is due to the isolated protein ions since these alone are capable of causing a Donnan equilibrium across a membrane impermeable to the protein ions but permeable to most crystalloidal ions.

3. The similar influence of electrolytes on the viscosity of protein solutions is due to the submicroscopic solid protein particles capable of occluding water since the amount of water occluded by (or the amount of swelling of) these particles is regulated by the Donnan equilibrium.

4. These ideas are supported by the fact that the more the submicroscopic solid particles contained in a protein solution or suspension are transformed into isolated ions (*e.g.*, by keeping gelatin solution for 1 hour or more at 45°C.) the more the viscosity of the solution is diminished while the osmotic pressure is increased, and *vice versa*.

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# THE NATURE OF FOVEAL DARK ADAPTATION.\*

By SELIG HECHT.†

(From the Marine Biological Laboratory, Woods Hole.)

(Received for publication, August 15, 1921.)

## I.

In a previous paper (Hecht, 1919-20, *c*) on the dark adaptation of the human eye, I attempted an analysis of the existing data on peripheral dark adaptation for the purpose of securing some hint of the physicochemical basis of visual reception. Early in the course of that analysis it became apparent that, aside from the deficiencies in the data themselves, there were lacking some rather elementary concepts on which to rest an objective treatment of the data. For example, there existed no notion of the relation between the intensity of the stimulating light and its photochemical effect in the retina. Moreover, it had apparently never been deemed necessary to entertain any ideas with regard to a mechanism with which to link up the variations in the sensitivity of the retina at different times.

Clear concepts of at least these two matters lie at the foundation of any attempt toward a rationally objective interpretation of the sensory behavior of the eye. It became necessary, therefore, to supply these defects in terms of what had fortunately been found in the studies of the photosensory responses of certain invertebrates (Hecht, 1921, *c*). As a result of these assumptions it was possible to analyze the data of dark adaptation of the eye, and to account for the phenomenon in terms of a comparatively simple photochemical mechanism.

The analysis was made on the best available data. These data were concerned with the periphery of the eye only. This condition

\* Part of the apparatus used in these experiments was secured by means of a grant from the Elizabeth Thompson Science Fund. To the trustees of this Fund I wish to express my thanks for the grant.

† National Research Fellow in Chemistry.

proved to be of advantage, because it opened the possibility of connecting the findings with what is known of the properties of visual purple. Moreover, the conclusions which were secured pointed to certain crucial experiments to be made with visual purple, thus suggesting a new mode of attack in this line of work (Hecht, 1920-21, *d*).

However, as far as the eye as a whole is concerned, the analysis was incomplete, because it did not include the properties of the fovea. Although the fovea is essentially an instrument for bright vision, and general dark adaptation, a phenomenon of dim vision, it still seems desirable to find out what occurs in the fovea during a stay in the dark. A study of the published data<sup>1</sup> shows that they are to a large extent insufficient and inconsistent, and that they involve some fundamental sources of error. It has therefore become necessary to investigate the matter from the beginning from a new point of view and with a new type of apparatus. I have done this, and the results show in a gratifying fashion that it was worth while. Not only has it been possible to find the facts and regularities, but it has been possible to account for the discrepancies of the results of other workers.

## II.

1. There are three major and several minor sources of error which enter into a study of dark adaptation. With but few exceptions previous work on foveal adaptation has been done without much consideration of the major sources, and with but scant attention to the minor ones. It is necessary to consider these possibilities of error carefully.

Measurements of the course of dark adaptation presume an original condition of light adaptation. It would seem obvious that this must be kept constant if the measurements are to have any significance. Still, this necessary starting point has been consistently disregarded, even when its significance has been recognized. We find that light adaptation is secured by such irregular means as reading in a room (*cf.* Dittler and Koike, 1912) or walking in the street (*cf.* Nagel and Schaefer, 1904). In some cases it is noted whether the day is cloudy or bright, but no account is taken of the circumstance.

<sup>1</sup> A review of the literature will be found in the papers by Tschermak (1902), Wölfflin (1910), and Dittler and Koike (1912).

A second source of error involves the change from light adaptation to darkness. It is again almost obvious that this change must be clean cut and accurately timed. In almost all of the previously published data this transition has involved the walking or running of the subject from the light-adapting location to the dark room. It follows that no measurements can be made during the variable interval of running or walking. Even if such measurements were possible, they would be of little significance for a study of the process, because one could not tell when dark adaptation actually began.

The third source of error involves the movements of the iris. At least two distinct things happen when a light-adapted eye is removed to darkness: the pupil dilates and the retina increases in sensitivity. In order that the data may be made to mean something, it is necessary to isolate the separate effects of these two factors.

The means which I used to study foveal adaptation have taken into account all these three sources of error. In addition I have considered certain precautions to limit the measurements to the foveal region. The entire procedure takes place in a large dark room. A constant condition of light adaptation is secured by having the subject look from a fixed distance at a brightly illuminated screen for a given interval. The change from bright illumination to complete darkness is made by shutting off the artificial source of illumination. The result is a clean cut transition. Simultaneously with this change, the subject is not even required to leave his seat. He merely raises his head 5 cm. when the lights are turned off, and is at once ready for measurement.

In the preliminary experiments I attempted to take care of the changes in the iris by the use of an artificial pupil. For several reasons I abandoned this method. First, it was rather clumsy, and proved to be a stumbling block in the accomplishment of a rapid change from the position of light adaptation to that in which the measurements were made. Second, the recent excellent experiments of Reeves (1918) made it possible to correct for pupil variation. And third, in consequence of the feasibility of this correction, it seemed desirable to secure the gross results. This would enable one to make comparisons with previous investigations, and would also give a correct notion of the total change in the eye as a whole, which is itself of some interest.

In order to confine the experiments to central vision, I utilized its characteristic perception of form and color. The object to be looked at was a red cross. The form was secured by a cross-shaped opening of such size that when looked at directly its image fell well within the rod-free area of the macula. The color was secured by a red filter. The measurements were determinations of the minimum illumination at which the subject could clearly see the object both as a cross and as a red one.

2. The essential parts of the assembled apparatus are given in Fig. 1, which shows three views of the arrangements. The screen for light adaptation is marked *N*. It is a closely woven linen screen similar to those used for white window shades. It is illuminated from behind by two Mazda lamps, *L*, 150 watts and 115 volts, backed by polished reflectors, and set 60 cm. from the screen. The switch for turning the lights on and off is in the hands of the experimenter. The subject looks at the region of the screen lying between the two lights; that is, between the two dashed circles in the front view of Fig. 1. The brightness of this field is 90 millilamberts, which is equivalent to an illumination of 0.028 candles per square centimeter of screen.

The construction of the apparatus for measuring the minimum intensity at which the red cross becomes visible is shown in partial section in the side view of Fig. 1. At *C* there is a sheet of opal glass. In contact with it, on the side facing the observer, is a Wratten Filter No. 70. This limits the transmission to the rays beyond  $650\text{ }\mu\mu$ , and thus insures the participation of the cones only. In front of the filter there is the opening already referred to. It is a cross-shaped opening in an opaque piece of cardboard. It is 24 mm. in diameter, each arm of the cross being  $8\times 8$  mm. The area of the cross is thus 320 sq. mm. The observer's eyes are placed next to the opening in the viewing box, above *P*. The appearance of this opening is seen in the front view. The distance from the eye to the cross is 55 cm. The cross therefore gives an image 0.65 mm. in diameter, which is about two-thirds the diameter of the rod-free area.

The illumination of the red cross is secured by means of a small microscope lamp situated in a lamp house, and properly surrounded by black screens. It is a 15 watt, 115 volt, concentrated filament, Mazda lamp, and may be considered as a point source for our purposes.

It rests on a movable carriage which rides freely along the bottom of the long rectangular blackened box shown in the figure. The carriage is only slightly narrower than the box, so that a proper and continuous centering of the lamp is insured. The light from the lamp falls on the opal glass, which diffuses it. It then passes through the filter,

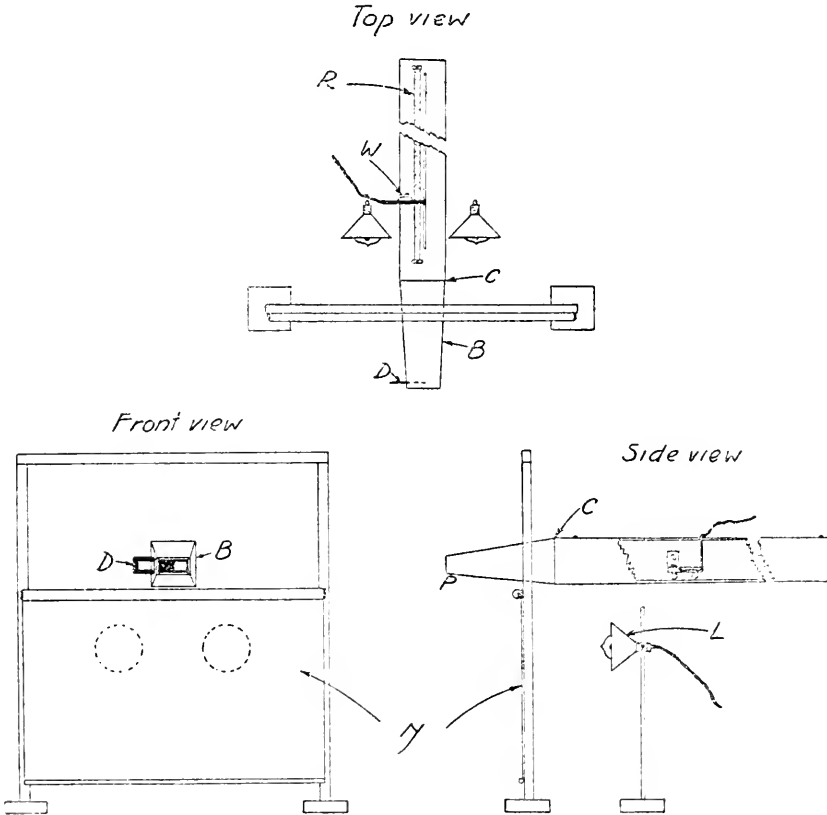


FIG. 1. Diagram of apparatus to secure constant light adaptation, and to measure the course of dark adaptation. An explanation is given in the text.

and goes to the eye. The illumination through the red cross may be varied by changing the distance of the lamp from the diffusing plate of opal glass. The intensity of the illumination emitted by the red cross is measured for a given position of the lamp, and the intensity at any other position calculated on the inverse square law. In these

experiments the lamp was always farther than 30 cm. from the opal glass; in this way a uniform illumination of the cross was assured.

The wires for the lamp run through a brass tube. This is rigidly set in the lamp carriage, and projects through a slit in the upper wall of the blackened box. The brass tube then turns a sharp right angle, and lies about a millimeter above the top of the box perpendicular to the long axis of the box, as shown in the top view of Fig. 1. Because of the rigidity of the construction, the lamp carriage can be easily moved by pushing on the outside arm of the brass tube. Moreover, the location of the lamp is in this way indicated by the position of the horizontal brass arm. An exact record of this position is made in the dark by running a pencil along the edge of the arm, and making a mark on the long strip of paper *R* which is stretched along the top of the box beneath the brass arm. The position of the brass arm indicative of a given location of the lamp is carefully measured. Its subsequent locations may then be read off with a meter stick from the established zero point.

The lamp is controlled by the experimenter by means of a noiseless switch *W* attached to the edge of the protruding brass arm.

### III.

The actual manner of making a series of measurements deserves some attention. With each subject I spent a preliminary period of about 30 minutes explaining the nature of the experiments and the construction of the apparatus. Aside from putting the subject into an interested frame of mind, this preliminary period serves as an experimental precaution. It allows time for the effects of the outside light, which is variable, to disappear. The starting point is therefore the same for all the experiments.

The subject is seated in a comfortable chair, the height of which is so adjusted that the eyes of the particular subject can look comfortably into the opening of the viewing box *B*. He then lowers his head, rests his forehead against the edge *P* of the box, and looks at the light-adapting screen. The subject keeps himself in a relaxed condition. To facilitate this, the chair is so placed as to give full support to the subject's back. Also, the edges of the viewing box are padded with black felt. 5 minutes are allowed for light



adaptation. Measurements made with regard to this point show that the process is accomplished in much less time (*cf.* Lohmann, 1906), but this interval is chosen to cover all contingencies. Near the end of the time the subject is given notice, and at the proper moment, the experimenter turns out the bright lights. The subject raises his head at once, and looks into the viewing box.

A few preliminary experiments showed at once that the rate of dark adaptation of the fovea is exceedingly rapid during the first minute of darkness. To make frequent and rapid measurements during this short interval is out of the question. Aside from the technical difficulties, there is the fact that repeated tests of the visual threshold must disturb the process of adaptation. The failure to appreciate this source of error has vitiated many of the experiments of Piper (1903) on peripheral adaptation, and nearly all of the results of Wölfflin (1910) on foveal adaptation. An ideal method would be to make but one reading following each light adaptation. This however would weary the subject, even if it did not fatigue the eye, which is not at all certain. The method finally adopted is a compromise between these two extremes.

Only one eye is used at a time in making the measurements. The eye to be used is under the control of the experimenter, who determines it by moving a slide *D* near the ocular end of the viewing box (Fig. 1). The subject acts as if he were looking with both eyes. Indeed, many of the subjects were unaware of which eye they were using, or that they were using only one eye; a few were able to distinguish them easily.

Before the bright lights are turned off, the small lamp is set at a given distance from the opal glass. When the lights are turned off, the subject looks in and announces the moment he sees the red cross. The time is taken by the experimenter with a stop-watch. The bright lights are turned on, the subject becomes light-adapted again, and the process is repeated with the other eye; this time the light has been placed nearer or farther depending upon the time it took the subject to see the cross. In this way after three or four trials the distance is determined at which the subject can see the red cross almost as soon as he looks into the box, immediately after the room has been darkened. The rate of dark adaptation at the beginning is so rapid

that it is impossible to measure this point accurately. The difficulty lies not so much with the determination of the intensity, as with the measurement of the exact time of dark adaptation. Half a second makes an enormous difference in the threshold, as will presently be apparent. However, a rough idea of the order of magnitude of the threshold after about 2 seconds dark adaptation can be secured in this way, and as such it is valuable.

All this time the subject has gained practice in locating the red cross. The experiment then begins in earnest. After the subject has again been light-adapted, the measuring lamp is set at such a place that the subject will see it after about 5 seconds in the dark. The slide is set for observation with the left eye, and the subject looks in as usual and announces when the red cross appears. The time is taken with a stop watch, the measuring lamp is turned out, and the position of the lamp marked on the strip of paper. The subject withdraws his head from the viewing box, and sits comfortably in the dark.

Readings of the stop-watch and observations of the time in the dark are made by a very dim light which is carefully screened from the subject. This dim light is turned on momentarily when it is needed by means of a spring contact controlled by the experimenter's foot. Repeated tests on this point have never revealed an instance when the subject was able to notice this illumination.

The slide in the viewing box is now adjusted for the right eye. The lamp is moved far back in the long box, and 2 minutes after the beginning of dark adaptation, the subject is requested to look into the viewing box. The measuring lamp is then turned on and brought nearer and nearer the cross opening at a rate of about 3 cm. per second. The subject then announces the moment he sees the red cross. The time is taken with a stop-watch which was started *exactly* 2 minutes after dark adaptation had begun. The lamp is turned off, its position is marked and numbered on the recording paper, and the subject is told to sit back comfortably in the dark. 6 minutes after the beginning of dark adaptation, the slide is set for the left eye, and a measurement made as in the last instance. The process is then repeated after 10 minutes of dark adaptation, this time with the slide set for the right eye. 15 minutes after the beginning of dark adaptation, a reading is made with the left eye again; and after 20 minutes adaptation,

with the right eye. It will be seen that observations with the same eye are made never less than 6 minutes apart. In the other three instances the interval is greater than that, being 8, 9, and 10 minutes, respectively.

After the last observation the bright lights are turned on, and the subject again becomes light-adapted for 5 minutes. The entire process described in the last three paragraphs is then repeated, except that the first observation is made with the right eye, the others following in alternating order. In this way an independent set of determinations is secured for each eye, reaching from the beginning to the end of dark adaptation. These six pairs of duplicate points are, however, not sufficient to map out accurately the entire course of dark adaptation. Therefore intermediate points are secured in the following way.

The subject is again light-adapted. The lights are turned off, and a determination is made with the left eye after about 30 seconds dark adaptation. The lamp is brought closer and closer in the usual manner until the subject announces the appearance of the red cross. The time is taken and the distance recorded. The subject rests in the dark, and after 3 minutes in the dark a measurement is made, this time with the slide set for the right eye. The bright lights are then turned on, the subject becomes light-adapted, and the 2 measurements are repeated, this time first with right eye for 30 seconds adaptation, and then with the left eye after 3 minutes dark adaptation.

It is not necessary to detail the method any further. Suffice it to say that two sets of ten determinations are secured, a set for each eye. The whole procedure may be shown in Table I, which gives the details of a single experiment. The table shows first, the order in which the observations are made; second, the period extending from the time when the bright lights were turned off, to the time when adaptation was announced; and third, the distance which the measuring lamp had to be brought before the subject announced that she saw a red cross. Each horizontal line in the body of the table indicates 5 minutes light adaptation. Thus, between two horizontal lines are the readings made during a single period of dark adaptation. An entire experiment takes about an hour and a half.

An observation is never verified immediately. Once the subject has announced that he sees the red cross, the lamp is at once turned off. If an observation is questioned either by the subject or by the experimenter, it is repeated later after a fresh light adaptation, and the subject is unaware of its repetition. The practice of verifying a reading within a second or so involves a number of fresh sources

TABLE I.  
*Record of a Single Experiment on Dark Adaptation of Fovea.*  
*Subject, M. G. ♀*

Left eye.			Right eye.		
Order No.	Duration of adaptation.	Distance.	Order No.	Duration of adaptation.	Distance.
	<i>min.: sec.</i>	<i>mm.</i>		<i>min.: sec.</i>	<i>mm.</i>
1	:06	352	2	2:15	1060
3	6:12	1410	4	10:15	1600
5	15:17	1345	6	20:10	1435
8	2:10	1100	7	:05	352
10	10:16	1355	9	6:12	1355
12	20:13	1480	11	15:10	1460
13	:32	635	14	3:12	1345
16	3:11	1235	15	:30	595
17	:16	470	18	1:01	880
20	1:02	805	19	:17	495

Horizontal lines between measurements indicate a period of 5 minutes light adaptation.

of error such as retinal fatigue and refractory period which are bound to influence the final result (*cf.* Cobb, 1919, p. 437). I, therefore, consistently avoided such a method.

#### IV.

1. After several preliminary experiments, I made a final set of measurements with fifteen subjects. Of these, eight were men and seven women. Their ages varied between 28 and 35 years. They were all of more than ordinary intelligence, all but one having been

university trained. Most of them were teachers; the rest were professional people acquainted with scientific matters. They were all interested in the experiments, and did all they could to follow instructions in the method of making observations.

The nature of the data which I secured with these people is well illustrated in Fig. 2, which gives the results of two experiments on the same subject made four days apart. Each point represents a single reading with one eye in the manner described. The ordinates

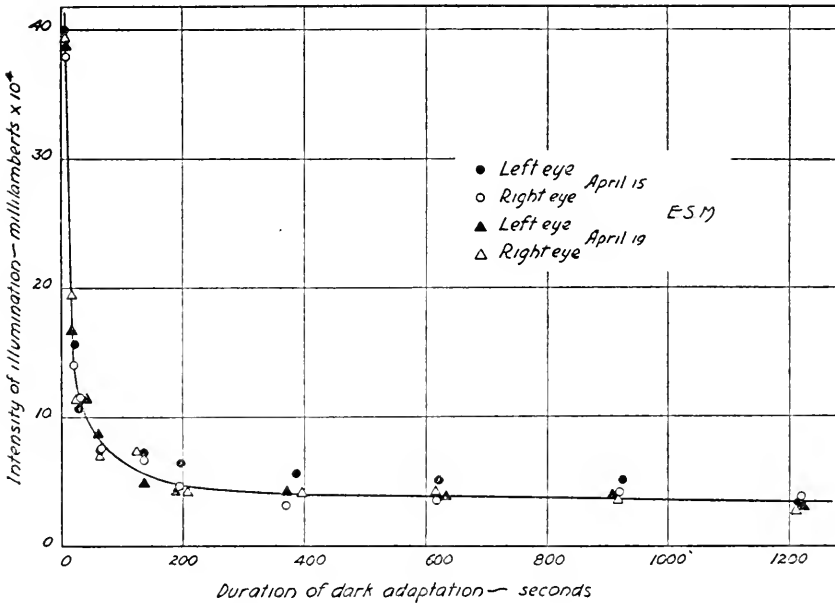


FIG. 2. Results of two experiments made 4 days apart with the same subject. The points are single readings as explained in the insert.

record the illumination emitted by the red cross when it just becomes visible. The data are not corrected for changes in the pupil area. The figure shows very clearly that the experiments were conducted under conditions that give reproducible results. I made a number of these duplicate sets on several individuals, and all show the same degree of coincidence in the position of the points.

In the results as a whole there is a certain amount of variation from individual to individual. One subject may be consistently

less or more sensitive than another. However, the shape of the curve of dark adaptation remains the same, and the degree of variation is not much more than would be expected from inspection of the points in Fig. 2. To show this, I have plotted in Fig. 3 the results of the first five subjects. The other ten subjects give exactly the same kind of data. Each point in Fig. 3 is the average of two readings, one for the left eye and one for the right. It is apparent

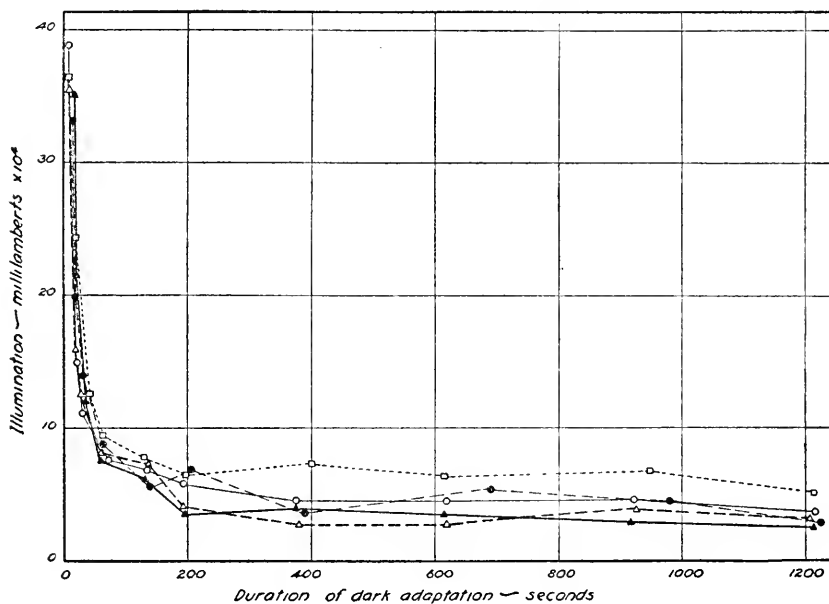


FIG. 3. Data for the first five subjects. Each point is the average of two readings, one for the left, and one for the right eye.

that the experiments are reproducible not only in a given individual, but in a group of individuals as well. In presenting the complete data I have therefore combined the measurements of the fifteen subjects, thus getting an average of thirty determinations of each of the ten points on the curve of dark adaptation. These average results are given in Fig. 4. It will be seen that the shape of the curve and the location of the average points are similar to those shown for the single points in Figs. 2 and 3.

2. One thing stands out prominently in all these figures. It is that the dark adaptation of the eye, as measured by foveal vision, begins immediately, and proceeds at a precipitous rate for the first 30 seconds. The data in Figs. 2, 3, and 4 begin to record the events which happen after the first 7 seconds in the dark. This is because things happen too fast for accurate measurement before the first 7 seconds. As noted in a previous section, however, I have made some observations of the threshold after 2 seconds of dark adaptation for

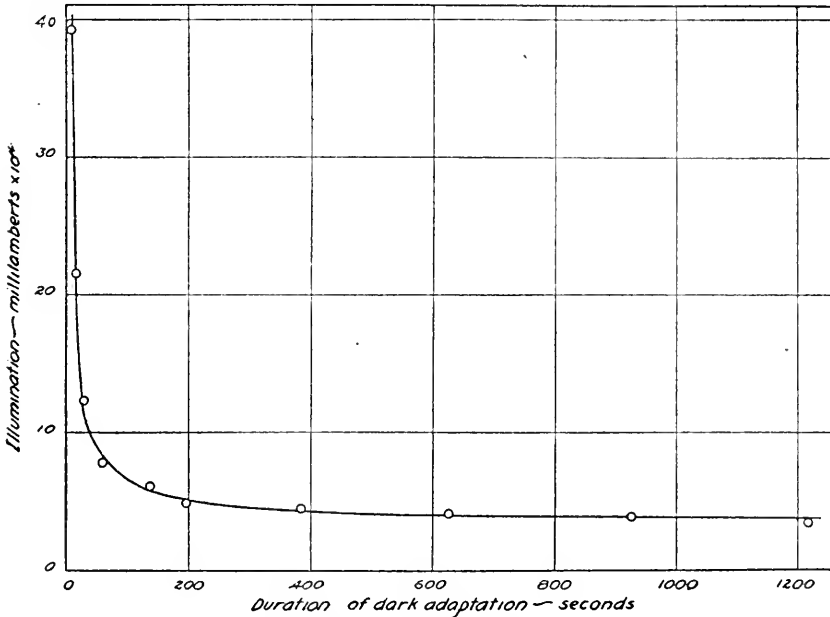


FIG. 4. Data for all fifteen subjects. Each point is the average of thirty measurements.

the purpose of securing an idea of the order of its magnitude. An average of twenty measurements gives this threshold a value of  $140 \times 10^{-4}$  millilamberts. From Fig. 4 it can be seen that the threshold after 7 seconds of dark adaptation is about  $40 \times 10^{-4}$  ml. After 30 seconds in the dark the threshold drops to about  $12 \times 10^{-4}$  ml. Because of the nature of the curve of dark adaptation, as described in a later section, it is possible to extrapolate backwards and find what the value of the threshold is at zero seconds dark adaptation. This turns out to be

$454 \times 10^{-4}$  ml. The threshold thus drops in the first 30 *seconds* to about 3 per cent of its initial value.

During the next 20 *minutes* the threshold further decreases, but only to about  $3 \times 10^{-4}$  ml., and remains there. It is therefore clear that the change which takes place after the first 30 seconds is comparatively insignificant in the light of the first rush of events. There is, however, no sudden point at which the process of adaptation begins to slow down. It slows down from the moment it begins, and as can be seen from the figures presented so far, its course is continuous and smooth.

#### v.

1. The precipitous course of foveal adaptation during the first few seconds is at the bottom of the curious results obtained by previous workers. It will not be irrelevant to consider in some detail the better and more recent of these investigations. Inouye and Oinuma (1911) studied foveal adaptation by the following method. A double tube was constructed through which the two eyes could look simultaneously at different fields. The brightness of the two fields could be separately controlled by a rotating sector of black and white cardboard. The idea was to keep one eye light-adapted, and the other dark-adapted, and then to vary the illumination in one tube until both eyes saw equally bright fields.

The actual procedure calls for a preliminary light adaptation of both eyes by reading in the laboratory near the dark room. One eye is then dark-adapted with a black bandage. After the proper interval the subject rushes into the dark room, tears the bandage from his eye, and looks through the two tubes. The sector wheel has been set, and the subject compares the brightness of the two fields. After repeated trials for the same amount of dark adaptation, the position of the sector is found which results in apparent equality of the two fields. The entire process is then repeated for different amounts of dark adaptation. These experiments were repeated by Dittler and Koike (1912), who used smoked glasses instead of a sector wheel with which to reduce the illumination of the dark-adapted eye.

The results secured by these two sets of investigators show a leisurely kind of dark adaptation for the fovea, Dittler and Koike's



data showing much the slower rate. For example, after 3 minutes adaptation the eye requires 22.5 per cent of the illumination of the light-adapted eye in order that both see the same brightness. After 30 minutes adaptation the ratio of light to dark-adapted eye is as 100 to 8. This represents total dark adaptation.

The explanation for this leisurely adaptation lies in the extreme rapidity with which adaptation actually proceeds during the first few seconds. Consider Dittler and Koike's method. One eye is light-adapted while the other is dark-adapted, and the standard of measurement is the light-adapted one. To get into the dark room takes a few seconds; to get to the apparatus takes a few more seconds; to remove the bandage, to accommodate, and to make a judgment also take a few seconds. During part of this time the light-adapted eye is in the dark, and as Fig. 4 shows, it is very rapidly becoming dark-adapted. When the comparison is being made, the light-adapted eye is no longer light-adapted. Its threshold is much lower than it was a few seconds ago. As a standard of comparison it is entirely too low, the situation being the same as if the measurement of dark adaptation were begun after the first few seconds have elapsed. This, together with the fact that the degree of light adaptation is low to begin with, could make the course of adaptation correspond with that portion of the curve in Fig. 4 which lies below, say, the middle of the scale of ordinates. This condition accounts for the apparent slowness of the process and for its small extent, both of which are characteristic of these and of the older investigations.

2. Considered from this point of view, these older results constitute a significant check on the validity of the experiments presented in the present paper. My measurements were made with red light, and it might perhaps be thought that the course of dark adaptation would be different if the experiments had been conducted with white light.

To meet this criticism I made a few experiments on my own eyes, using red light at one time and white light at another. Although the actual threshold illumination is not the same in the two cases, the rate of adaptation is the same. This matter has already been considered by Nagel and Schaefer (1904, p. 283), who came to the same conclusion. They used red, blue, and green lights and found that the same order of change is secured with all three colors. In other words, the Purkinje phenomenon does not exist in the fovea.

Moreover, the experiments which I have just reviewed, though differing in accuracy from mine, are indeed roughly comparable to them when properly evaluated, as I have pointed out above. They show, for example, that foveal adaptation practically ceases after 10 minutes in the dark. In addition, if the value of the light-adapted eye as a standard be tripled or quadrupled, as it must at least be because of the initial speed of adaptation, the final degree of dark adaptation represents about 1 per cent of the condition of the light-adapted eye. This is practically the same value which is found in the present experiments. Calling the initial threshold  $454 \times 10^{-4}$  ml., the final threshold of  $3.4 \times 10^{-4}$  ml. represents a drop to 0.8 per cent. Therefore the results with red light show the same course of adaptation as experiments with any other light. A careful analysis of our present data will thus be of significance for the general problem of adaptation and foveal vision.

## VI.

The data as they stand include the results of two phenomena: the movement of the iris, and the change in the fovea. In order to isolate the effects due to the foveal changes alone, it will be necessary to correct the data for variation in the pupil size. If the pupil area did not vary, the energy received by the retina would depend on the intensity of the illumination. On the other hand, if the intensity were to remain constant, the energy transmitted to the retina would be directly proportional to the varying area of the pupil. It then follows that since both intensity and pupil area vary, the energy received by the retina is proportional to the product of the intensity and the area.

We have measured the intensities; it is therefore necessary to introduce the data for the area of the pupil. These can be supplied from the experiments made by Reeves (1918). The experimental situations in the two cases are comparable. Reeves determined the changes in the pupillary diameter in darkness following adaptation to artificial illumination of about 100 ml. In my experiments the light adaptation field had a brightness of 90 ml. These illuminations result in practically the maximum effect in constricting the pupil.

In Fig. 5 are given the results calculated from the diameter values in Table IV of Reeves' paper. From the smoothed curve it is simple to find the pupil areas corresponding to the points plotted in Fig. 4. It is not necessary to correct for the effect of the cornea on the apparent size of the pupil, because we are interested in the relative variation in the pupil area. Calling unity the area of the pupil after 7.1 seconds dark adaptation, the first point in Fig. 4, I have computed the relative areas of the pupil for the different points in Fig. 4. These values are given in Table II.

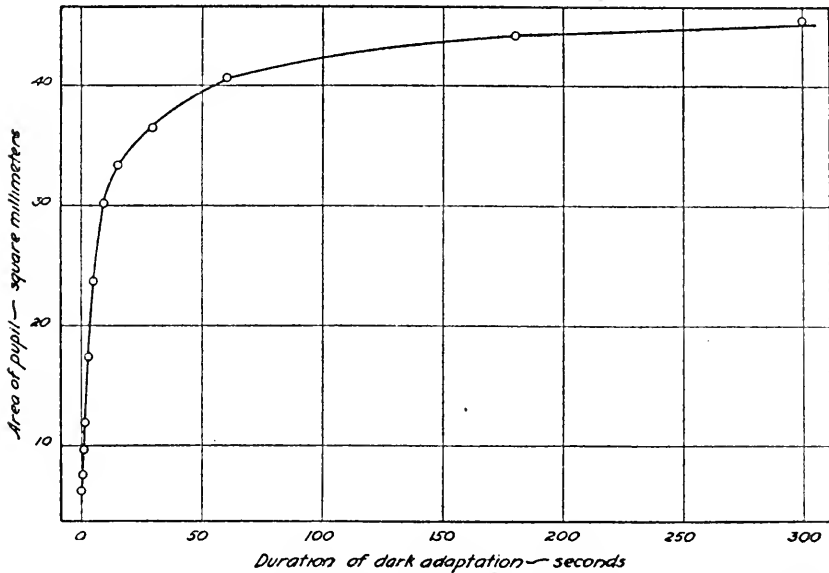


FIG. 5. Change in the area of the pupil during dark adaptation. The data are computed from the diameters given in Table IV of Reeves' (1918) paper.

In the last column of the same table are given the values of the products of the measured intensity into the relative area of the pupil. These numbers represent the intensity thresholds at different times in the dark on the assumption that the pupil area has maintained a constant area equal to its value at 7.1 seconds. This point is chosen arbitrarily because it is the first accurately measured point in these experiments. Any other point would have done just as well, because all that we are concerned with are the relative changes at constant

TABLE II.

*Relation between Apparent Illumination, Area of Pupil, and Corrected Illumination.*

Duration of adaptation.	Observed illumination.	Relative area of pupil.	Corrected illumination.
<i>sec.</i>	<i>ml. <math>\times 10^4</math></i>		<i>ml. <math>\times 10^4</math></i>
7.1	39.30	1.00	39.30
16.4	21.60	1.19	25.70
29.3	12.30	1.28	15.80
59.5	7.81	1.47	11.50
136.0	6.09	1.56	9.50
196.0	4.78	1.58	7.57
385.0	4.49	1.62	7.28
626.0	4.06	1.63	6.62
925.0	3.86	1.63	6.30
1217.0	3.37	1.63	5.50

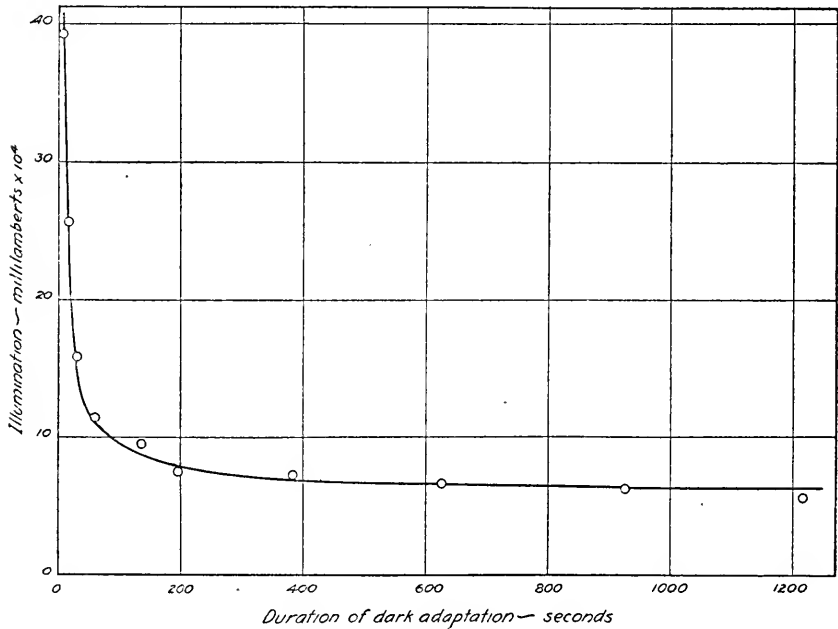


FIG. 6. Dark adaptation of fovea independent of pupil changes. The points are those in Fig. 4 corrected in terms of Fig. 5. They represent the illumination necessary to stimulate the fovea assuming a constant pupil whose area is 27.1 square millimeters. This is the area at 7.1 seconds dark adaptation, and corresponds to the first point in Figs. 4 and 6.

pupil diameter. These results are plotted graphically in Fig. 6, and their values are indicative of the changes which take place in the fovea independent of the movements of the iris. It is to the interpretation of these data that we shall now turn our attention.

## VII.

In considering the action of light in vision, it is hardly an assumption to suppose that the first effect of the light is the photochemical decomposition of a sensitive substance. Our measurements represent this effect in terms of the minimum intensity of illumination required to produce a visual sensation at different times in the dark. What is the relation between the intensity of the stimulating light and its objective photochemical effect? In the analysis of peripheral adaptation (Hecht, 1919-20, *b*) I assumed that the photochemical effect is proportional to the logarithm of the intensity. The reasons for this assumption were first, that such a relationship had been found in the clam, *Mya*; and second, that the gamut of change undergone during peripheral adaptation was so enormous that a logarithmic relation is the only one that would bring the data into a form amenable to ordinary comprehension and analysis. These reasons are not imperative. They derive their final justification on the pragmatic ground that the assumption of a logarithmic relation results in the formulation of a simple chemical picture which accounts for the data satisfactorily.

The range of dark adaptation in the fovea is not a tithe of the extent encountered in the periphery. The older data indicate a change from 10,000 to 1 in the minimum intensity necessary for a peripheral effect. Cobb's (1919) more recent experiments, as well as my own unpublished ones, indicate even a greater change than this. The older measurements were faulty in a number of ways, particularly in their neglect of the first few seconds of adaptation. Compared to these changes, those presented here for the fovea are really small in extent. When corrected for constant pupil diameter, the necessary minimum for a response after 2 seconds of foveal adaptation is about  $70 \times 10^{-4}$  ml. The extrapolated value at zero becomes, after pupil correction,  $103 \times 10^{-4}$  ml. After 20 minutes the intensity as given in Fig. 6 is about  $6 \times 10^{-4}$  ml. This range of change represents noth-

ing startling, it being the kind ordinarily encountered in chemical processes. In treating the data further I have therefore assumed that the photochemical action of the light is directly proportional to the intensity. This is a common enough situation in photochemical reactions, and in addition, obtains in the photic sensitivity of *Ciona* (Hecht, 1918-19, *a*).

It follows from Fig. 6 that the quantity of sensitive material decomposed in order to produce a threshold effect in the fovea gradually but consistently decreases during dark adaptation. What is the basis for this variation? In other words, with what chemical or physical entity or process in the fovea can this gradual decrease be associated? I have previously outlined a possible hypothesis to account for this, and because of its success in the study of the responses of *Ciona* and *Mya* as well as of the periphery of the human eye (Hecht, 1921, *e*), I venture to suggest its application in the present circumstance.

Let us consider what might conceivably happen in the fovea. We have accepted the existence of a photosensitive substance. During light adaptation this substance must be decomposed to a considerable extent. Associated with this is a decided increase in the threshold of stimulation. During the succeeding period of dark adaptation there is produced a characteristic decrease in the threshold which proceeds according to a definite pattern. During this time it seems reasonable to suppose that the sensitive material is in some way being formed again, and that its concentration in the sense cells is increasing.

The continuously decreasing threshold cannot be directly associated with this increase in sensitive material. One reason for this is the fact that they vary in the opposite sense. A more cogent reason is the nature of the sensitive material itself. In its essence it must be an inert material so far as the sensory process is concerned. Otherwise it would continually be initiating visual effects. It is only after it has been changed into something else by the light, that it produces its characteristic reaction.

Indeed, it is to this decomposition product that we must look for some objective visualization of the process of dark adaptation. This decomposition product undoubtedly enters into some secondary reaction. I have elsewhere suggested that in *Mya* and *Ciona* it serves to catalyze a second reaction, the end-products of which constitute

the "inner stimulus" for the nerve endings. It is not necessary at present to be specific on this point. It is enough to accept the general idea that the decomposition product is the active agent in the early stage of the formation of the visual impulse. Its effects, catalytic or otherwise, will undoubtedly be a function of its concentration in the sense organ. An increase in this concentration will cause an increase in its effect. It would, therefore, seem a reasonable assumption to make that the increase in concentration necessary to produce a perceptible effect in the secondary reaction is directly proportional to the concentration already present. This is the "compound interest law" which is at the bottom of so many physical and chemical phenomena.

The assumption then is that in order to produce a photosensory effect, the light must cause an increase in photolytic products proportional in extent to the concentration already present in the sense organ. Such an assumption has served successfully in the analysis of the photosensory process in *Mya* and *Ciona* and in the periphery of the human eye. Moreover, in the case of *Ciona* it was possible to put the matter to a critical experimental test, the results of which completely vindicated the assumption (Hecht, 1918-19 *a*, p. 162).

#### VIII.

In the case of the present data as given in Fig. 6, this assumption plus the one relating intensity and photochemical effect lead to this. The ordinates, representing the intensity of the threshold, are directly proportional to the amount of photolytic products formed by the light. These values are in turn directly proportional to the concentration of photolytic products already present in the sense organ. The curve of dark adaptation of the fovea therefore represents the changes which take place in the concentration of the photochemical decomposition products in the sensory mechanism.

It is apparent that this concentration is decreasing steadily. This decrease can represent one of two possible processes: either the products are diffusing out of the sensory cells, or they are reacting chemically in the formation of some other substance. The nature of the curve in Fig. 6 will be of some aid in deciding between these alternatives.

The relation between the intensity  $I$  and the photochemical effect  $E$  is linear, and is represented by the equation of a straight line

$$E = k I + b$$

The constant  $k$  will depend on the units employed; it may therefore be made equal to unity by changing the units. The constant term  $b$  represents the intercept on the  $y$  axis. In the present case, omitting the factor  $10^{-4}$ , it turns out to have a value of  $-6.0$ .

It is then necessary to find what the relation is between the photochemical effect  $E$ , representing the concentration of photolytic products, and the time  $t$  during which the eye has been in the dark. Calculation shows this relation to be that of a bimolecular chemical reaction

$$k = \frac{1}{at} \times \frac{x}{a-x}$$

in which  $k$  has a value of 0.00271.

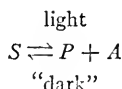
In the calculation of the data according to the bimolecular isotherm,  $a$  is taken as the value of the first point in Fig. 6. This makes  $a$  equal to  $39.3 - 6.0$ , or 33.3 units. Correspondingly,  $t$  is taken as zero at 7.1 seconds, and the other values of  $t$  are computed accordingly. This avoids the use of any highly uncertain values of the threshold at the first movement of dark adaptation. Once the constant  $k$  has been determined in this way, it is possible to calculate the real value of  $a$  at  $t=0$ . This extrapolation from the experimental results and the above equation gives  $a$  a value of 103.2 units. It should be noted that the curve in Fig. 6 is drawn from values calculated from the equation of a bimolecular reaction in which  $k$  has the value already given.

Several things follow from the fact that the curve is that of a bimolecular reaction. One is that the gradual disappearance of the decomposition products is not a process of diffusion. Under certain conditions the rate of diffusion may be a linear function of time. More normally it is a function of concentration. In the latter event its course is represented by an equation similar to that of a monomolecular reaction. A bimolecular reaction isotherm represents not a process of diffusion, but one of chemical reaction only.



Granting the chemical nature of the process, it is obvious that there exist two products of decomposition which are disappearing from the reaction mixture by combining to form something else. Concomitant with this, in the same sense cells, there is being produced an increasing concentration of photosensitive material. What more reasonable step is there than to assume that it is being formed by the combination of the decomposition products that are disappearing?

We are therefore led to the conclusion that in the photosensory mechanism of the fovea there exists the following arrangement of materials. There is a photosensitive substance  $S$  whose rate of photolysis at threshold intensities is a linear function of the intensity. The photolysis of this material results in the formation of two products of decomposition  $P$  and  $A$ . The concentration of these decomposition products at any moment determines the sensitivity of the sensory mechanism, in that the amount of photolytic action necessary for a threshold, visual effect is directly proportional to the concentration of photolytic products already present. Finally, these decomposition products constantly recombine to form fresh sensitive material, their combination proceeding according to the kinetics of a bimolecular reaction. We have thus the familiar reversible reaction



in which the products of decomposition serve as the precursors of the sensitive material.

#### IX.

It is not necessary at this time to dilate on the simplicity with which such a reversible system accounts for the observed facts (*cf.* Hecht, 1919-20, *c*, p. 514). It will suffice to point out that the "dark" reaction obviously accounts for dark adaptation; that the stationary state, in which the "dark" and light reactions are balanced, takes care of the condition of sensory equilibrium in which the eye has become adapted to a given light intensity; and that the process of light adaptation represents merely the displacement of the stationary state of the reaction to the right, due to the increased action of the light.

It is worth drawing attention to the fidelity with which the calculated curve of Fig. 6 adheres to the observed facts of dark adaptation. For further testimony Table III is presented showing this agreement in the usual manner. Considering all the things which enter into the experiments and into the calculations, the agreement seems striking.

This agreement, however, need not be considered an infallible *experimentum crucis*. It is possible that some other formula might fit the facts just as well. However, the significance which attaches to the agreement is that it follows from a reasoned construction of what may

TABLE III.

*Comparison of the Observed Values for the Threshold during Dark Adaptation with Those Calculated in Terms of a Bimolecular Reaction.*

Duration of adaptation.	Threshold intensity.	
	Observed.	Calculated.
<i>sec.</i>	<i>ml. × 10<sup>4</sup></i>	<i>ml. × 10<sup>4</sup></i>
7.1	39.3	(39.3)
16.4	25.7	24.1
29.3	15.8	17.0
59.5	11.5	10.9
136.0	9.5	8.6
196.0	7.6	7.9
385.0	7.3	7.0
626.0	6.6	6.6
925.0	6.3	6.4
1217.0	5.5	6.3

take place in the cone cell of the fovea. The circumstance that a similar type of photochemical mechanism differing in local detail of application serves to account for the photosensitive behavior of *Mya*, and for that of the peripheral retina, heightens the plausibility of the general picture.

With regard to these local differences in the treatment of the general idea there are several interesting points to be considered. I shall indicate just one of these. For the periphery (rods) of the eye the application of the principle of a reversible reaction depends on the assumption of a logarithmic relation between intensity and photo-

chemical effect. For the fovea (cones), however, a similar interpretation depends on a linear relation between intensity and photochemical effect. What constitutes the basis of this difference between rod and cone will be a nice point if it is ever established. I have already suggested (Hecht, 1919-20, *b*) that the logarithmic relation may hinge on some absorption phenomenon associated with the sensory mechanism. However, a detailed treatment of this and other possibilities is not appropriate at this juncture, and will be reserved for a later occasion.

The general idea which I have suggested as underlying all these phenomena of photic sensitivity has an immediate use. In the case of *Mya* it has served as a means of suggesting fresh and crucial experiments that have already contributed materially to its support. In venturing to suggest a similar mechanism for the eye, I hope that it may help to point the direction in which further work may be done. It would seem that by such a method progress may be made in the rather chaotic field of visual physiology. A concrete picture, though it limits itself to the initial events in vision, should serve better than the vague, sweeping theories of which there are already far too many.

#### SUMMARY.

1. After a discussion of the sources of error involved in the study of dark adaptation, an apparatus and a procedure are described which avoid these errors. The method includes a control of the initial light adaptation, a record of the exact beginning of dark adaptation, and an accurate means of measuring the threshold of the fovea after different intervals in the dark.

2. The results show that dark adaptation of the eye as measured by foveal vision proceeds at a very precipitous rate during the first few seconds, that most of the adaptation takes place during the first 30 seconds, and that the process practically ceases after 10 minutes. These findings explain much of the irregularity of the older data.

3. The changes which correspond to those in the fovea alone are secured by correcting the above results in terms of the movements of the pupil during dark adaptation.

4. On the assumption that the photochemical effect of the light is a linear function of the intensity, it is shown that the dark adaptation

of the fovea itself follows the course of a bimolecular reaction. This is interpreted to mean that there are two photolytic products in the fovea; that they are disappearing because they are recombining to form anew the photosensitive substance of the fovea; and that the concentration of these products of photolysis in the sense cell must be increased by a definite fraction in order to produce a visual effect.

5. It is then suggested that the basis of the initial event in foveal light perception is some mechanism that involves a reversible photochemical reaction of which the "dark" reaction is bimolecular. Dark adaptation follows the "dark" reaction; sensory equilibrium is represented by the stationary state; and light adaptation by the shifting of the stationary state to a fresh point of equilibrium toward the "dark" side of the reaction.

Dr. W. E. Forsythe of the Nela Research Laboratories was kind enough to calibrate the illumination emitted by the red cross and by the light-adapting screen. I take pleasure in thanking him for his courtesy and help in this connection.

I also wish to express my appreciation of the gracious cooperation of my various friends who served as subjects in what must have been a tedious performance. I feel particularly indebted to those who were sufficiently interested and kind to serve as subjects for duplicate and triplicate experiments.

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# THE SELECTIVE ABSORPTION OF POTASSIUM BY ANIMAL CELLS.

## II. THE CAUSE OF POTASSIUM SELECTION AS INDICATED BY THE ABSORPTION OF RUBIDIUM AND CESIUM.

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That the peculiarities of the physiological behavior of potassium may be related to its ionic mobility has been suggested by Bayliss (1). Loeb (2) has referred its peculiarities to its electronic structure; *i.e.*, to the outside ring of electrons and its atomic number. These, in accord with modern views, would determine hydration. Hydration, according to Jones (3), is responsible for the difference in ionic velocities of members of the group of alkali metals. The arrangement of outer electrons and the atomic number then may be regarded as determining the relative ionic mobility. This is the general explanation as set forth by Mines (4) and others as to why ionic mobilities have a complex relationship to ionic volumes. The fact that potassium is generally selected in preference to sodium by a great variety of living cells is especially notable among its several peculiarities. Is there any evidence to show that the difference in the migration velocities of potassium and sodium are concerned in this so called "selective action"?

Among the comparative ionic conductances of the univalent metallic ions, together with the ammonium ion which has similar behavior, we note certain relations. The figures below are taken from Landolt (5) and refer to ionic conductances at 18°C.

Li	Na	NH <sub>4</sub>	K	Rb	Cs
33.4	43.5	64	64.6	67.5	68

The interval between Na and  $\text{NH}_4$  is much greater than intervals between the others. Lithium and sodium may be regarded as constituting one group, while ammonium, potassium, rubidium, and cesium fall into another. These are all univalent ions so that their absolute velocities of migration show the same relations as their conductances. If, then, the absorption of potassium by cells in preference to sodium is related to comparative ionic mobilities, lithium should behave like sodium, but rubidium and cesium like potassium. In a number of other physiological processes, just such relationships have been shown. Loeb (2) has pointed out how lithium and sodium stand in one group, physiologically speaking, while ammonium, potassium, rubidium, and cesium clearly constitute a distinctly different group. The members of each group show among themselves a number of similarities in physiological behavior, while the effects of the two groups may be regarded, in some ways, as physiologically divergent. Because of the manner in which hydration affects ionic mobilities the migration velocities of the members of this series seem to provide a basis for grouping them in accordance with their physiological behavior more satisfactorily than do other periodic functions, as for example, atomic volumes. We have sought to show whether or not the contrast of physiological properties among this series holds for their intracellular incorporation. We have not yet made a study of lithium. There is much in the literature on the subject to indicate that lithium is capable of penetrating into cells with no greater facility than sodium. The behavior of ammonium ions is obviously without much bearing on the question of potassium selection, since they freely pass in and out of all cells and are to be regarded, in general, as a waste product and not as constituents of cellular architecture. The experiments herein reported go to prove that rubidium and cesium behave like potassium in processes involving incorporation into the physicochemical structure of muscle and other animal cells.

Mendel and Closson (6) found that rubidium injected into cats and dogs was largely stored in muscle tissue. Zwaardemaker (7) and his coworkers in researches on aquiradio activity have shown that rubidium and in some degree cesium can replace potassium in maintaining the heart beat, but whether this means penetration into cells or action at their surfaces it is difficult to say. In the light of



our observations we would conclude that it shows the former to be true even though the effect upon irritability may be at the cell surface. So far as we have been able to read Zwaardemaker's reports in the original we have found no evidence on this aspect of the question and in this connection our results seem of some significance.

#### EXPERIMENTAL.

We perfused frog muscles with a Ringer solution modified by the replacement of potassium chloride with an aequimolar concentration of rubidium chloride. While both legs were perfused the muscles of one were made to contract by stimulation of the lumbar plexus with maximal tetanizing induction shocks lasting 1 second at 30 second intervals during one-half hour periods with alternating one-half hour periods of complete rest. In one experiment this procedure was continued during 5 hours and was followed by perfusion with an isotonic cane sugar solution during  $1\frac{1}{2}$  hours. All the muscles of both legs showed irritability at the end of the experiment. Samples of the gastrocnemius and sartorius of each leg were then decomposed in a mixture of nitric and sulphuric acids. The resulting solutions enabled us to detect rubidium spectroscopically in the muscles of the stimulated leg but not in those of the other. In another similar experiment the muscles of one leg were given nine half-hour work periods, that is, 540 contractions of 1 second each, and were then while resting, perfused during 2 hours with a potassium-free Ringer solution. The muscles of both legs showed good irritability at the end of the experiment. The wet-ashed muscle samples, taken from the gastrocnemius and vastus muscles of each leg, were made up to 10 cc. and examined spectroscopically by Gooch's method for quantitative estimation of rubidium. No trace of rubidium could be detected in the muscles perfused without stimulation. The samples used were 1.27 gm. from the gastrocnemius and 2.37 gm. from the vastus. In the muscles of the stimulated legs, however, we found approximately 0.011 per cent of rubidium. The standard solution for spectroscopic comparison with the muscle material contained 3 per cent of  $\text{H}_2\text{SO}_4$ , 0.075 per cent of  $\text{KCl}$ , 0.025 per cent of  $\text{NaCl}$ , and 0.0025 per cent of rubidium added in the form of the carbonate. It was designed to imitate, approximately, the acid, potassium and

sodium content of the solutions of wet-ashed muscle. The samples taken from the stimulated muscles were 1.86 gm. from the gastrocnemius, and 2.03 gm. from the vastus. The standard solution diluted 3.5 times gave the faintest rubidium spectrum possible to detect. The solution from the gastrocnemius behaved similarly when diluted 3 times and the one from the vastus when diluted 3.5 times. A careful uniformity of technique was used in making all the flame tests. This estimation shows 0.011 per cent of rubidium in the gastrocnemius and 0.012 per cent in the vastus. As a matter of fact a proportion of rubidium larger than these figures indicate was present because the muscles were obviously edematous. Rubidium content, in proportion to the dry solids, was not estimated. An amount of rubidium, equivalent to more than 3 per cent of the normal potassium content

TABLE I.  
*The Absorption and Retention of Cesium by Frog Muscle*

Volume of cesium solution used.	Duration of first perfusion.	Volume of potassium-free Ringer solution used.	Duration of second perfusion.	Weights of samples of muscle used for analysis.				Cesium found.	
				Right gastrocnemius.	Right vastus.	Left gastrocnemius.	Left vastus.	Left gastrocnemius.	Left vastus.
cc.	hrs.	cc.	hrs.	gm.	gm.	gm.	gm.	per cent	per cent
625	7	530	4½	2.80	2.78	2.57	2.37	0.008	0.006
475	6½	500	3½	3.01	2.39	2.55	2.97	0.006	0.006

of frog muscle, was taken up when the muscles made 540 contractions of 1 second each. The retention of rubidium in the muscles during perfusion with a rubidium-free solution and the absence of rubidium from the unstimulated muscles, points to its actual entrance into and incorporation with the cell substance in the same sense that potassium is normally held there. Whether or not it actually replaces potassium has not yet been determined.

Similar experiments were made with cesium chloride, replacing, in equimolar concentration, the potassium chloride of Ringer solution. The data of two such experiments are presented in Table I. The muscles of the left leg of the frog used in each experiment made 420 contractions of 1 second each, while the cesium-containing solution was perfusing through both legs. The muscles of the right leg were meanwhile at rest. At the end of the experiment the muscles of

both legs showed good response to both direct and nerve stimuli; though, to the same stimulus, muscles of the right leg responded, of course, more vigorously than those of the left. Adequate samples of muscles of the right legs (amounts of samples are recorded in the table) showed no trace of cesium in a careful spectrum analysis. Muscles of the left legs showed brilliant cesium spectra, permitting a quantitative estimation. Cesium, then, like rubidium was taken into the muscle substance so as to be retained, in part, during the subsequent perfusion with potassium-free Ringer solution.

To further test the replaceability of potassium by rubidium and cesium young white rats were fed on purified synthetic diets in which salts of rubidium or cesium were substituted for those of potassium as ordinarily used in such diets. The basal diet had the composition shown below.

	<i>per cent</i>		<i>per cent</i>
Casein.....	18	Dried yeast.....	5
Starch.....	54	Salts.....	5
Butter fat.....	18		

In the experiments with rubidium each 5 gm. of salt mixture contained approximately:

	<i>gm.</i>		<i>gm.</i>
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.55	$\text{CaH}_4(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ .....	0.54
$\text{NaH}_2\text{PO}_4 \cdot 4\text{H}_2\text{O}$ .....	1.10	Calcium lactate.....	1.40
RbCl.....	1.29	Iron lactate.....	0.12

In the cesium experiments each 5 gm. of the salt mixture contained approximately:

	<i>gm.</i>		<i>gm.</i>
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.46	$\text{CaH}_4(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ .....	0.45
$\text{NaH}_2\text{PO}_4 \cdot 4\text{H}_2\text{O}$ .....	1.27	Calcium lactate.....	1.12
CsCl.....	1.59	Iron lactate.....	0.11

Water was given freely and to it a few drops of iodine solution were added once a week. The animals were put in separate cages, weighed frequently and kept under observation. After varying periods (10 to 15 days) the rats showed marked symptoms of derangement. We noted a sluggishness and general inactivity, followed by a period of marked irritability, with trembling and intermittent twitching which later amounted to tetanic spasms. Within 48 hours after the first tremblings were noted

the rats died in violent tetanic spasms. As a control, the above diet with potassium chloride in amounts equivalent to the rubidium or cesium chlorides was fed to rats with satisfactory results although the same diet plus rubidium or cesium chloride was fatal with the usual symptoms. Moreover a diet in which only one-half of the potassium was replaced by an equivalent amount of cesium or rubidium was lethal. In other words the rubidium and cesium in quantities used were toxic, irrespective of the presence or absence of potassium. The toxicity of rubidium and cesium was more pronounced than we had expected from the literature dealing with a variety of biological experiments in which these salts have been employed. We have found that as little as 2 cc. of 1.44 per cent solution of rubidium

TABLE II.

*The Absorption of Rubidium and Cesium by Rat Tissue.*

Weight of rat at beginning of diet.	Duration of feeding.	Kind of diet.	Weight at end of experiment.	Rubidium or cesium content of tissues weighed fresh.					
				Muscle.	Heart.	Liver.	Kidney.	Spleen.	Lungs.
gm.	days		gm.	per cent	per cent	per cent	per cent	per cent	per cent
55	17	Rb	50	0.33	0.19	0.22	0.21		
40	17	Rb	39	0.49	0.23	0.34	0.21		
53	13	Cs	57	0.120	0.042		0.060	0.055	0.035
56	10	Cs	46	0.043	0.034	0.039	0.038	0.036	0.017
70	10	Cs	82	0.538	*	0.430	0.555	*	0.219

\*Richly present as shown by spectrum. Amount not measured.

chloride (a concentration isotonic with 0.7 per cent sodium chloride), injected into the dorsal lymph sack of frogs weighing from 30 to 40 gm., killed within 24 hours, while 1 cc. on two successive days was also lethal. Autopsy of the rats revealed nothing of note except in one case showing an infection of the lungs. Various tissues of some of the rats which had been on diets containing rubidium or cesium were decomposed in a mixture of sulphuric and nitric acids. The resulting clear solution was made up to 10 cc. and subjected to spectrum analysis for estimation of rubidium or cesium. Results are summarized in Table II. We satisfied ourselves that these metals were actually contained in the cells by a thorough perfusion through a cannula in the left ventricle of a rat, chloroformed after 10 days on the cesium-containing diet. Potassium-free Ringer solution was

employed. The tissues of this animal showed a high content of cesium.

The considerable quantity of rubidium or cesium in the rat muscle, as shown in some of the experiments, is striking. Computed in actual concentration, it amounts in three of the experiments to about half the concentration of potassium as given for normal mammalian muscle (0.32 to 0.42 per cent). This looks like an actual replacement of potassium by rubidium or cesium. We have not yet undertaken the difficult and somewhat uncertain methods for determination of potassium in the presence of such quantities of the interfering substances, rubidium and cesium. The spectroscope, beyond revealing qualitatively that some potassium was present, would not serve the purpose. We cannot, therefore, draw any conclusions as to whether rubidium and cesium are taken into the cell in the place of potassium or in addition to it. It is interesting to note that rubidium and cesium can replace potassium with considerable success in furthering excitability as studied by Zwaardemaker (7) and his coworkers, in antagonistic salt reactions as shown by Loeb (2), and in permitting the development of *Arbacia* eggs as shown by Loeb (8). All of these effects may be due, although proof is not entirely adequate, to action at cell surfaces. In contrast to this, a prolonged action of rubidium and cesium, under circumstances permitting their incorporation into the cell would seem from our observations to constitute physiologically a less successful substitution for potassium.

#### SUMMARY.

1. Frog muscles perfused with Ringer solution in which potassium chloride has been replaced by an equivalent amount of rubidium or cesium chloride take up rubidium or cesium and incorporate them into the tissue substance in such form as to be retained during a subsequent perfusion with potassium-free Ringer solution, provided the muscles contract during the first perfusion. Retention of rubidium or cesium by a resting muscle does not occur.

2. Rats on synthetic diets, adequate in all respects except that potassium was replaced by an equivalent amount of rubidium or cesium, died after a period varying from 10 to 17 days with characteristic symptoms including tetanic spasms. Muscle, heart, liver,

kidney, spleen, and lung tissues were then found to contain significant amounts of rubidium or cesium. The concentration of these metals in the muscle amounted, in some cases, as shown by a spectroscopic estimation, to about half the concentration of potassium normally found in mammalian muscle.

3. The results are regarded as tending to confirm the theory that the peculiarities in the physiological effects of potassium, including the facility with which it is "selected" by living cells in preference to sodium, are related to the electronic structure of the potassium ion as compared with that of similar ions. The possible relationship of the comparative migration velocity, a function of the electronic structure, to physiological effects is suggested.

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# THE RELATION OF RESPIRATION TO RHYTHM IN THE CARDIAC GANGLION OF *LIMULUS POLYPHEMUS*.\*

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A study of the carbon dioxide production by the cardiac ganglion of *Limulus polyphemus* has shown a definite relation between the rate at which the neurogenic heart beats and the intensity of the respiration of the nerve cells which develop the rhythm. This has been demonstrated by the parallelism between the temperature coefficient of rate of heart beat and of CO<sub>2</sub> production,<sup>1,2</sup> and also by the striking diminution in the CO<sub>2</sub> produced by the nerve cells when the inhibitory nerve to the ganglion is stimulated—a fact which correlates the inhibitory action of the ganglion with the respiratory processes of its nerve cells. These facts at once suggest that automatic impulse formation by the cardiac ganglion of *Limulus*, and probably by all automatic nerve centers, may be determined by the velocity of those chemical reactions in which carbon dioxide is produced by the nerve cells.

The following report presents additional evidence in support of this thesis and shows that a variety of agencies which stimulate the cardiac ganglion and increase the rate of heart beat, produce a similar increase in the rate of CO<sub>2</sub> production by the nerve cells of the heart ganglion.

## Method.

The method was the same as that used in previous work.<sup>1,2,3</sup> The color change of phenolsulfonephthalein was used to determine the

\* Funds granted by the American Association for the Advancement of Science were used to meet the expenses of this investigation.

<sup>1</sup> Garrey, W. E., *J. Gen. Physiol.*, 1920-21, iii, 41.

<sup>2</sup> Garrey, W. E., *J. Gen. Physiol.*, 1920-21, iii, 49.

<sup>3</sup> Garrey, W. E., *J. Gen. Physiol.*, 1920-21, iii, 163.

rate of change in hydrogen ion concentration which resulted from the formation of  $\text{CO}_2$  by the excised cardiac ganglion. The ganglionic cord was immersed in 3 cc. of a standard non-buffer balanced saline solution in small Pyrex glass tubes. The initial pH was 7.8 and the time required to reduce the alkalinity to pH 7.4 was used as an index of the rate of  $\text{CO}_2$  formation. To facilitate manipulation the ganglion was draped over glass hooks on a non-soluble glass rod which was cemented into the paraffined cork used to stopper the indicator tube. The standard immersion solution was made by adding 2.2 cc. of  $\text{m}/2$   $\text{CaCl}_2$  to 100 cc. of  $\text{m}/2$   $\text{NaCl}$  and the desired initial pH, 7.8, was secured by adding the requisite amount of sodium hydroxide.

#### EXPERIMENTAL RESULTS.

*Electrical Stimulation.*—Faradic stimulation of the posterior end of the cardiac ganglion always produces acceleration of the beat of the intact heart. The excised immersed ganglion was similarly stimulated by means of platinum electrodes passed through the cork of the indicator tube. The rate of change in the  $\text{CO}_2$  production was compared under this treatment with that of the unstimulated ganglion and the results indicate that during stimulation the rate of  $\text{CO}_2$  production is enormously increased, being at least doubled or trebled as shown in Table I. This result is unquestionably due to increase in the chemical processes in the nerve cells, for the faradic shocks do not produce the color changes in the solution, even when the electrodes dip directly into the solution or when applied to a narcotized ganglion. The decrease in  $\text{CO}_2$  production, when the inhibitory nerve to the ganglion is stimulated, also forms a control experiment which supports the conclusion that we are concerned with a true stimulation of the processes of respiration in the nerve cells in question.

*Mechanical Stimulation. Stretching.*—A further check upon the results of faradization is obtained by stretching the nerve cord. Carlson<sup>4</sup> had found that distension of the *Limulus* heart cavity increased the rate of the heart beats by its mechanical effect upon the cardiac ganglion. That stretching the nerve cord might produce

<sup>4</sup> Carlson, A. J., *Am. J. Physiol.*, 1907, xviii, 149; *Ergebn. Physiol.*, 1909, viii, 423.



distinct chemical changes seemed likely from analogy with a stretched striated muscle—which increases its osmotic pressure,<sup>5,6</sup> and produces excess of both lactic acid<sup>7</sup> and CO<sub>2</sub>.<sup>8</sup>

To determine the rate of CO<sub>2</sub> production by the cardiac ganglion when stretched, silk ligatures were attached to either end of the nerve cord and looped over hooks on the glass mounting rod. By means of these threads it was possible to stretch the ganglion repeatedly, increasing its length some 20 per cent of normal, without apparent injury. The rates of CO<sub>2</sub> production before, during, and after the stretching in five experiments are given as averages in

TABLE I.  
*Faradic Stimulation and Rate of CO<sub>2</sub> Production.*

Condition of ganglion.	Time required to change pH 7.8 to pH 7.4.				
	Experiment No.				
	1	2	3	4	5
	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>
Normal.....	192 221 218	266 268	327 332 315	840	1620
During stimulation.....	81 100	130 168	137	263	255
After stimulation.....	238 229 222	310 275	367 342	907	1620

Table II. That the increase during stretching is not merely a result of increasing surface for diffusion, is indicated by the persistent increase in the rate of CO<sub>2</sub> production after the stretching operation had stopped. It is due to the mechanical stimulation of the respiratory rate; and the results thus run parallel to the increase in the rate of heart beat when the ganglion is similarly stretched.

<sup>5</sup> Cooke, E., *J. Physiol.*, 1898, xxiii, 137.

<sup>6</sup> Garrey, W. E., *J. Biol. Chem.*, 1909, vi, p. x.

<sup>7</sup> Gotschlich, E., *Arch. Physiol.*, 1894, lvi, 355.

<sup>8</sup> Eddy, N. B., and Downs, A. W., *Am. J. Physiol.*, 1921, lvi, 188.

*Alcohol.*—In concentrations of one-half to one per cent by volume the cardiac ganglion of *Limulus* is markedly stimulated by ethyl alcohol<sup>9</sup> and may double the rate of heart beat. These concentrations of purified absolute alcohol, when added to the standard sodium-calcium chloride mixture do not of themselves modify the color of phenolsulfonephthalein used as pH indicator. In these concentrations, however, there results an easily demonstrable increase in the rate of CO<sub>2</sub> production by the ganglion, a result which is again in concord with the interpretation that the increased rate of heart beat depends upon the increased respiration in the nerve cells of the ganglion. Table III records the results of some of the experiments with ethyl alcohol as the stimulating agent.

TABLE II.  
*Rate of CO<sub>2</sub> Production by Stretched Ganglion.*

Condition of ganglion.	Average time to change pH 7.8 to pH 7.4.				
	Experiment No.				
	1	2	3	4	5
	sec.	sec.	sec.	sec.	sec.
Normal.....	200	228	117	251	375
Stretched.....	142	170	77	163	263
Immediately after stretching.....	168	200	206	236	318
15 minutes after stretching.....	190	208		245	335

*Sodium Chloride.*—In the isotonic concentration, M/2, a sodium chloride solution is a pronounced stimulus to the automaticity of the cardiac ganglion of *Limulus*. The rate of heart beat is promptly accelerated and the individual beats of the muscle merge until a tetanic condition is induced by the continuous discharge of impulses from the ganglionic cells. Determinations of the rate of CO<sub>2</sub> production by the ganglion immersed in M/2 NaCl showed an increase in the rate of respiration of the nerve cells which harmonizes with expectations based on the increase in rate. The rate of CO<sub>2</sub> production was tremendously increased. The averages found in experiments with six ganglia immersed in isotonic NaCl gave the

<sup>9</sup> Carlson, A. J., *Am. J. Physiol.*, 1906, xvii, 177.

following percentages of the normal rate; *i.e.*, 311 per cent, 218 per cent, 237 per cent, 145 per cent, 260 per cent, 134 per cent. Subsequent to these determinations immersion of the ganglia in the sodium-calcium solution demonstrated the antagonism of these ions in their effects on animal oxidations for the mixture caused a return of the rate of respiration to within 15 per cent of the previous normal in every instance. This fact excludes the possibility that the increase in CO<sub>2</sub> formation in the isotonic NaCl is a lethal phenomenon, in the ordinary sense of that term, or at least that the stages dealt with

TABLE III.  
*Effects of 1 per cent Ethyl Alcohol on Rate of CO<sub>2</sub> Production.*

Condition of ganglion.	Time required to change pH 7.8 to pH 7.4.					Average rate of CO <sub>2</sub> production.
	Experiment No.				Average time of all experiments.	
	1	2	3	4		
	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>per cent</i>
In Na/Ca solution (normal).....	184	265	315	448	305	100
In Na/Ca + 1 per cent ethyl alcohol.	110	150	145	245	160	190
	90	140	150	265		
	102		165	290		
In Na/Ca solution.....	130	148	230	485	296	103
	147		255	230		
	201	248				
In Na/Ca solution + 1 per cent alcohol	152	166	160	315	198	154

are still reversible (Osterhout)<sup>10</sup>. Loeb and Wasteneys<sup>11</sup> working with concentration of sodium chloride isotonic with sea water found a decrease in oxidation in eggs of *Arbacia* and of *Gonionemus*; this apparent disharmony with the behavior of *Limulus* heart ganglia may be specific for the material used or may be merely an expression of the duration of exposure to NaCl or of the concentrations of the sodium chloride to which the tissue is subjected since Brooks<sup>12</sup> has

<sup>10</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1920-21, iii, 15.

<sup>11</sup> Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1910, xxviii, 340; 1911, xxxi, 168.

<sup>12</sup> Brooks, M. M., *J. Gen. Physiol.*, 1919-20, ii, 5.

shown that one can get an increase or a decrease in the respiration of *Bacillus subtilis* depending upon the concentration of NaCl employed.

*Adrenalin Chloride.*—Addition of one drop of adrenalin chloride solution (1 part to 10,000 parts of solvent) to each cubic centimeter of the standard sodium-calcium solution stimulates the ganglion and increases the heart rate<sup>9</sup>. Experiments with three ganglia immersed in this concentration of the drug gave the average rate of CO<sub>2</sub> production as 204 per cent, 194 per cent, and 166 per cent of the normal.

These experiments amply justify the generalization that those stimuli, which acting on the cardiac ganglion to cause an increase in the rate of the heart beat, likewise produce changes which result in a marked increase in the respiratory processes within the nerve cells. Impulse formation by the ganglion, and CO<sub>2</sub> production are therefore intimately related processes and appear to stand in relation of effect and cause respectively. A complete justification of this postulate requires that we shall be able to establish a quantitative correspondence between the processes of impulse formation, that is rate of heart beat, and the rate of CO<sub>2</sub> formation. Experiments directed to this end could not be conducted in such a way as to record both the rate of heart beat and the rate of CO<sub>2</sub> production on the same preparation simultaneously, but it was possible to secure a satisfactory approximation by taking advantage of the characteristic effects produced by changes of temperature especially the effects of transient exposure to relatively high and low temperatures. These are considered in the following paragraphs.

#### *Effects of Temperature Extremes (Transitory Exposures).*

Reference has been made in previous communications<sup>1,2</sup> to the fact that if the ganglion is exposed for a few minutes to a very low temperature (0°C.) and then warmed to 10°C., for example, a new rate of rhythm is established which is faster than was obtained at this temperature before cooling. This increase in rate may amount to 25 per cent or 30 per cent. A similar effect upon the rate of carbon dioxide produced by the ganglion has been noted under like treatment, and eight examples have been referred to in Tables I and II of a former paper.<sup>2</sup> In four experiments more recently carried out the times required for the color change in our indicator at 10°C.

were 408, 532, 618, and 803 seconds, but after exposing the ganglia for five minutes to 0°C. then again warming to 10°C. the time was in each case shortened being respectively 320, 416, 482, and 629 seconds. The increase in the rate of CO<sub>2</sub> production averages 28 per cent in these experiments and is in good quantitative correspondence to the acceleration rate of rhythm as stated above.

When on the other hand ganglia are exposed to the upper extremes of temperature compatible with function, *e.g.*, to 35°C. or 40°C., and are subsequently cooled the rate of rhythm is much *slower* at the lower temperature than before warming process; thus in five experiments in which the rates at 25°C. were determined before and after heating the ratios of the actual rates of beat were 20:17, 20:13, 18:13, 24:20, and 17:8. This corresponds to an average decrease to 70 per cent of the previous rate. Four other experiments in which similar conditions obtained indicated a depression in the rate of CO<sub>2</sub> to a point only 78 per cent of the value before exposure to the high temperature. These values for both extremes of this temperature range again show a very good quantitative correspondence between the effects upon rate of heart beat and rate of CO<sub>2</sub> formation by the ganglia.

The quantitative relationship between the two processes is made much more striking by the graph given in Fig. 1. This gives the curve for a single typical experiment in which the rates of beat are plotted as ordinates against temperature as abscissæ. In this experiment the ganglion was progressively cooled from room temperature to 0°, then warmed to 38°, and again cooled. The direction of the temperature change is indicated by the arrows and the rate by outline characters, triangle, circle, and square, respectively.

A companion graph was constructed for the rate of CO<sub>2</sub> formation. For this purpose eleven experiments were taken in which the ganglia had been treated to the same progressive changes in temperature as in the previous experiment on rate of heart beat. The average time required for CO<sub>2</sub> formation was determined for different temperatures. It was found that if the rate of CO<sub>2</sub> formation was expressed as the reciprocal of the time in seconds and this was multiplied in each case by the constant 1400 a plot of the results gave a curve identical with that shown in Fig. 1. The points obtained by this calculation

have been introduced into Fig. 1 as solid characters. This striking quantitative parallelism between rate of heart beat and rate of development of  $\text{CO}_2$  in the cardiac ganglion, which is brought out by this graph, forcefully substantiates the probability that the two

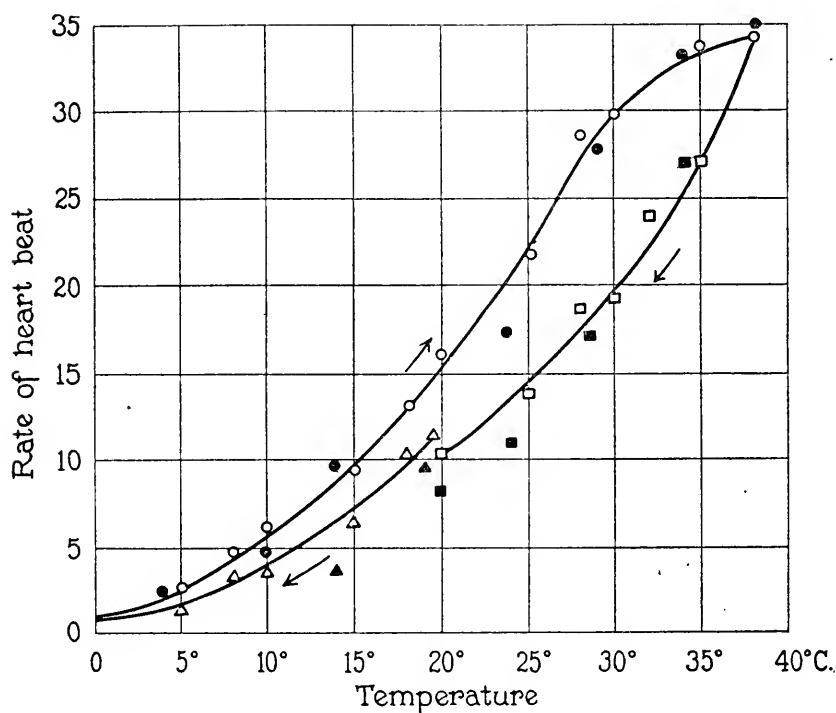


FIG. 1. Curve of rate of heart beat when the ganglion is initially cooled from the normal temperature to  $0^{\circ}\text{C}$ . ( $\Delta$ ), then progressively warmed to  $38^{\circ}\text{C}$ . ( $\circ$ ), and finally cooled to room temperature ( $\square$ ). The solid characters indicate the corresponding average rates of carbon dioxide production by the ganglion.

physiological processes are bound together in the relationship of cause and effect and that it is the velocity of those chemical reactions which give rise to carbon dioxide formation in the cells of the cardiac ganglion which determines the rate of the neurogenic heart of *Limulus polyphemus*.

## COMPARATIVE STUDIES ON RESPIRATION.

### XIX. A PRELIMINARY STAGE IN THE PROGRESS OF ETHER ANESTHESIA.

By EDITH PHILIP SMITH.

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(Received for publication, September 1, 1921.)

One of the fundamental aspects of the action of the anesthetics is their effect upon respiration. In order to study this under conditions where the anesthetic is the only variable, it is desirable to employ plants, since experiments with animals are usually complicated by muscular action and its influence on the rate of respiration. Under suitable conditions the rate of respiration in plants remains remarkably constant for periods of time considerably longer than the average experiment, so that they offer good material for this type of work.

The method employed in the writer's experiments was that described by Osterhout.<sup>1</sup> The material used was wheat, which was chosen because it is easy to grow, and gives very constant results. The wheat was a pure strain, supplied by the Minnesota Experimental Station, and was particularly well suited to the purpose, being uniform in growth and showing a high viability. The seeds were germinated with aseptic precautions, being grown in sterile paper cups in a saturated atmosphere in the dark. The greatest care was necessary to avoid contamination by molds, and the most effective way of checking them was found to be that of soaking the dry seeds for at least 10 minutes in full strength commercial hydrogen peroxide before germinating. Cultures showing the slightest sign of molds were rejected, as they gave aberrant results. It was found that by allowing no liquid water in the cultures after the roots had begun to emerge, but by keeping the atmosphere saturated (by means of a bell jar inverted over the Petri dish full of water in which the paper cups stood), the seeds produced an abundance of very well-developed

<sup>1</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1918-19, i, 17.

root-hairs. The material was used when the roots were from one to one and a half inches long, with abundant surface for absorption and respiration. Before using, the seeds were washed for 10 minutes in running water. They were then put into a flask of Pyrex glass, with 100 cc. of distilled water, and the apparatus was allowed to run. After the rate of respiration had become constant (in distilled water) it usually remained so for at least 8 hours. As the average experiment lasted from 2 to 4 hours, it was possible to discount any variations in the rate as being due to manipulation, provided that the temperature remained constant. During the course of an experiment the temperature seldom varied more than a degree either way. The average room temperature was 20°C.

The indicator used was phenolsulfonephthalein in aqueous solution. The normal rate of respiration was taken as the reciprocal of the time required to change the pH value of the indicator from pH 7.36 to pH. 7.09, these values being chosen as being sufficiently different in tint to be easily read. The time varied with the age of the seedlings, ranging between 30 and 60 seconds.

The seeds were treated with a watery solution of ether, the concentrations employed being 1 per cent, 3.65 per cent, and 7.3 per cent (by volume). After the rate of respiration had become constant, 100 cc. of the required solution was substituted for the distilled water in the flask, and the experiment continued.<sup>2</sup>

With a solution of 1 per cent ether in distilled water the first effect was a fall in the rate of respiration. Fig. 1 (average of five experiments) shows that the minimum reached was 36 per cent of the normal, 6 minutes after the beginning of exposure to the ether. The rate then rose gradually towards normal, but remained below it for 48 minutes. The next stage was a rapid rise to 124 per cent, the maximum being reached in 51 minutes, and the duration above normal being 6 minutes. This was succeeded by a depression, when the rate fell gradually to about 50 per cent in about 4 hours.

With a solution of ether 3.65 per cent the first effect was the same, the rate reaching a minimum of 48 per cent in 9 minutes. The depression lasted for 24 minutes, and was followed by a rapid rise, the

<sup>2</sup> Care was taken to avoid any possible errors due to the admission of air in opening the apparatus or to the introduction of distilled water.



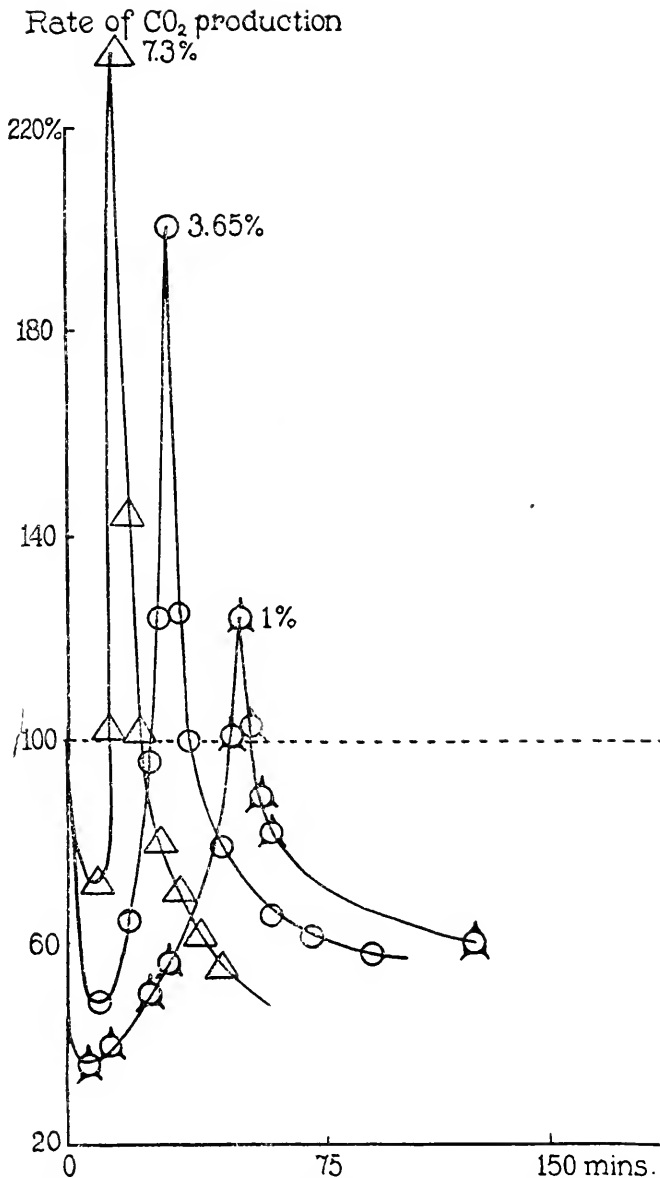


FIG. 1. Curves showing the rate of respiration of wheat seedlings (expressed as per cent of the normal), in 1 per cent, 3.65 per cent, and 7.3 per cent ether (by volume). The normal rate (which is taken as 100 per cent), represents a change in pH value from 7.36 to 7.09 in a number of seconds varying with the age of the material, usually between 30 and 60 seconds. The curve with 1 per cent ether represents the mean of five typical experiments (probable error of the mean, less than 10 per cent of the mean). The curve with 3.65 per cent ether represents the mean of ten typical experiments (probable error of the mean, less than 5 per cent of the mean). The curve with 7.3 per cent ether represents the mean of six experiments (probable error of the mean, less than 5 per cent of the mean, except for two points, where the error is 10 per cent of the mean).

rate reaching a maximum of 200 per cent in 30 minutes after beginning the exposure to the ether. This second rise undoubtedly masked the earlier fall in experiments by the older methods, which did not allow readings to be made at such short intervals. The rise is succeeded by a fall, so that after 78 minutes the rate was reduced to 60 per cent. The rate continued to decline slowly after this. The above figures represent the mean of ten typical experiments.

With 7.3 per cent ether, the general effects are similar. A minimum rate of 70 per cent of the normal was reached in 9 minutes, and the respiration remained subnormal for 12 minutes. The succeeding rise was very rapid, and reached a maximum of 235 per cent in 15 minutes. The rate then fell rapidly, reaching 60 per cent in 39 minutes. These data are the mean of six typical experiments.

In order to determine if the observed preliminary fall was really due to the action of the ether, and not to some experimental error inherent in the method, the work was repeated, using the direct indicator method as developed by Haas. The seeds were placed in a Pyrex tube of the same dimensions as the standard buffer tubes used for comparison, and the normal rate determined by adding measured quantities of tap water, containing phenolsulfonephthalein, 5 drops to 10 cc. The water was brought to the required pH value by the addition of traces of NaOH. The tube was closed, and inverted several times, so as to mix the contents thoroughly. After the time taken to change from pH 7.36 to 7.09 was noted, the seeds were well rinsed with several changes of tap water. The ether was made up with tap water and the indicator added. It was found that the results were essentially the same. That is, a curve was obtained showing three well marked divisions: a preliminary fall, a rise, and a final slower declension. It is thus reasonably certain that these results are due to some specific action of the ether.

The increase in rate followed by a fall was also observed by Thomas<sup>3</sup> in experiments on wheat but she does not describe a preliminary depression. In regard to the latter the results recall those of Irwin<sup>4</sup> on frog eggs and *Fundulus* embryos. In these cases Irwin found that the first effect of ether, in certain concentrations, was a fall in the rate

<sup>3</sup> Thomas, H. S., *J. Gen. Physiol.* 1918-19, i, 203.

<sup>4</sup> Irwin, M., *J. Gen. Physiol.* 1918-19, i, 209.

of respiration. The significance of these results seemed to be doubtful, and no emphasis was laid on them. The present experiments on plants, therefore, indicate that this preliminary depression may be

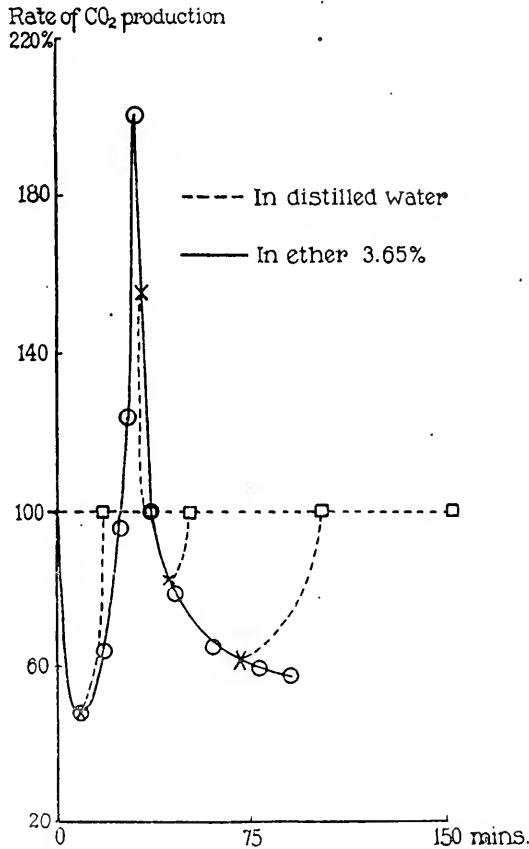


FIG. 2. Curves showing recovery from exposure to ether. Normal rate as in Fig. 1. The solid line represents respiration in ether; the broken line, respiration in distilled water. The points marked  $\times$  on the curve indicate points of removal from ether. The curve represents the mean of six experiments (probable error of the mean, less than 5 per cent of the mean).

of general occurrence. In that case their importance is evident, and must be reckoned with in any attempt at a general theory of anesthesia.

The interpretation of these results raises several questions. For example, which part of the curve represents stimulation, which anesthesia, and at what point do the toxic (irreversible) changes begin? In regard to the latter, it was found that, even when the respiration had been reduced below normal, complete recovery was possible on removal to distilled water, provided that the depression was not too great (Fig. 2). Below a certain range the respiration fell even more rapidly when the seeds were removed from the ether. There was no evidence of incomplete or partial recovery.<sup>5</sup> It is thus seen that up to a certain point, which may be below the normal rate of respiration, the curve represents changes in the rate of carbon dioxide production which are completely reversible. The boundaries of the stimulatory and anesthetic effects must be left for future experiments to decide.

#### SUMMARY.

Using these concentrations of ether (1 per cent, 3.65 per cent, 7.3 per cent), the following conclusions may be drawn.

1. The first effect of ether is to cause a depression in the rate of respiration. This is followed by a rapid rise above normal, which in turn is succeeded by a fall.
2. With all these concentrations the respiration is ultimately reduced to approximately the same level; the stronger the ether, the less time required to produce this result.
3. Even when the respiration has been reduced below normal, recovery is possible on removal from the ether, and appears to be complete, if sufficient time is allowed. If, however, the rate has been too far depressed, no recovery is possible.
4. These results extend those of Irwin on frog eggs and *Fundulus* embryos.

<sup>5</sup> These experiments were made by Osterhout's method.<sup>1</sup>

# STEREOTROPIC ORIENTATION OF THE TUBE FEET OF STARFISH (ASTERIAS) AND ITS INHIBITION BY LIGHT.\*

By A. R. MOORE.

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(Received for publication, Sept. 19, 1921.)

Recently Maxwell has shown that the reactions of sharks to contact stimuli are due to changes in the relative tension of the antagonist muscles similar to those taking place in the galvanotropic, heliotropic, and geotropic reactions of animals.<sup>1</sup> The writer has recently made observations on the starfish which show that contact stimuli applied to the sides of a ray bring about changes in the orientation of the tube feet which are comparable to heliotropic reactions. These stereotropic reactions of the starfish gain especial significance since they can be inhibited by the reaction to light.

In order to demonstrate the stereotropic orientation of the tube feet, the starfish is laid on its back in a dish of sea water. If the animal is prevented from righting itself for 1 or 2 minutes it becomes comparatively quiet; if now a contact stimulus is applied to one of the rays by pressing a foreign body such as a piece of cork, a glass rod, or a finger tip against the side of the ray, a retraction of the tube feet and closure of the ambulacral groove occurs. Next, the groove opens and the tube feet move toward the stimulated area. The reaction is especially marked in the immediate vicinity of excitation but in the more sensitive individuals it involves the entire ray. The average length of time which elapses between the moment of contact excitation and the protrusion of the tube feet is 2.8 seconds. The contact stimulus may be applied momentarily and removed before the reaction begins, but the series of reactions proceeds as

\* The experiments described in this paper were done in the Botany Laboratory at Woods Hole, and the writer wishes to express his thanks to Professor Osterhout for many courtesies extended to him during the progress of the work.

<sup>1</sup> Maxwell, S. S., *J. Gen. Physiol.*, 1920-1921, iv, 19. von Uexküll, J., *Z. Biol.*, 1900, xxxix, 73.

described. This gives confirmatory proof of the machine-like character of the reaction. It is also worthy of note that the circumoral nerve ring plays no part in the reaction, but only the radial nerve through which the tube feet receive their impulses since these experiments can be made on isolated rays. Fig. 1 is a diagram showing the orientation of the tube feet which are extended toward the point of contact stimulation.

If two points on the same side of the ray but at a distance from each other are touched, then the tube feet turn to that side (Fig. 2). The tube feet midway between the two loci of stimulation bend neither toward the one nor toward the other but at right angles to a line

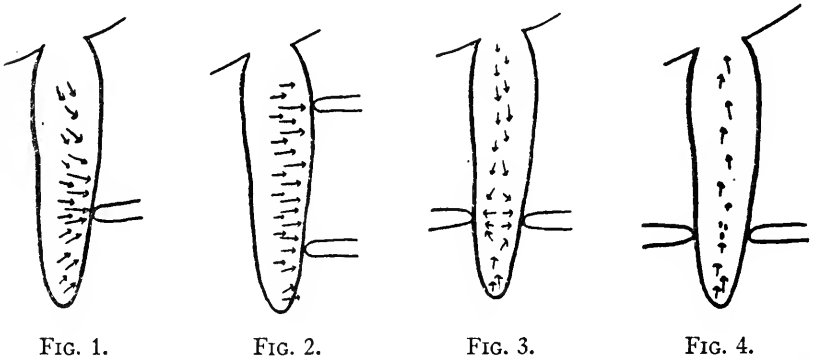


FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 1. Arrows indicate the direction in which the tube feet are bent, the arrow tip being put for the terminal disks. This diagram shows the tube feet all inclined accurately to the locus of contact excitation indicated by the U-shaped outline.

FIG. 2. Two loci of contact excitation on the same side of the ray result in the tube feet orienting themselves at right angles to the axis of the ray and to a line joining the two loci of stimulation. The median tube feet do not incline to either point of excitation but orient like a phototropic insect placed midway between two lights of equal intensity.

FIG. 3. Slight contact excitation has been applied at two points on opposite sides of the ray. Only the tube feet in the immediate vicinity are directed toward the points of excitation. The other tube feet are directed along the median axis of the ray at right angles to a line joining the two loci of stimulation.

FIG. 4. The same as in Fig. 3, except that the pressure is strongly applied resulting in withdrawal of the tube feet in the immediate vicinity of excitation, and orientation of the others to the center parallel with the axis of the ray and perpendicular to a line joining the two loci of excitation.

joining the two points. In this we have a tropistic reaction analogous to that of heliotropic orientation to two sources of light.<sup>2</sup> The same principle may be illustrated in another way by gently pressing the ray between two small bodies such as glass rods. When the extension reflex takes place it will be seen that only a few of the tube feet bend laterally and they are in the immediate vicinity of the points touched. All the other tube feet bend along the axis of the ray toward the area of excitation, swaying a little from side to side, but neither markedly to the right nor to the left (Fig. 3). If the pressure is increased the tube feet central to the point of stimulation reverse their orientation and bend toward the center (Fig. 4). However, not every animal gave both phases of this reaction; *i.e.*, distal and central bending of the tube feet. In the main the results were similar to those obtained by Maxwell with *Mustelus* in which he found that weak mechanical stimulation caused bending toward the point of contact while strong stimulation produced the opposite result.<sup>1</sup> It happens therefore that when acted upon by contact on two opposite sides of the ray, *i.e.*, by two equally balanced impulses, the tube feet orient themselves along a line perpendicular to a line joining the two loci stimulated. Here again is a case analogous to that of heliotropic orientation to two sources of light, since the starfish ray like the heliotropic insect is bilaterally symmetrical with reference to right and left.

If the tube feet as a result of their extension in response to contact touch a surface, they at once adhere by means of their sucking disks. When a considerable number of tube feet have thus taken hold it is difficult to pull the animal away from a surface. Even if one succeeds in doing so some of the tube feet will be torn from the animal and left sticking to the surface, so strong is the hold they have upon it. It is, however, possible by means of the light reaction to cause adhering starfish to release their hold. This reaction may be demonstrated as follows: A starfish is placed ventral side up in a dish of sea water in a dimly lighted room. As soon as the tube feet have been thrust out, a flash of sunlight is thrown across the animal. As a result the tube feet withdraw and the ambulacral

<sup>2</sup> Loeb, J., *Forced movements, tropisms, and animal conduct*, Philadelphia and London, 1918, 75. Patten, B. M., *J. Exp. Zool.*, 1914, xvii, 213.

grooves close; the rays bend ventrally. After several seconds in this position the grooves open and the tube feet are extended. This occurs even if the illumination is continuous.

For purposes of better control all the experiments with light were made in the dark room. While the retraction is uniformly elicited in the dark-adapted starfish with white light of sufficient quantity; red light has no such effect. It was therefore practicable to observe the animals at any time by means of red light while white light was admitted by a shutter for any desired length of time. Use was made of the lamp and optical bench described by Hecht.<sup>3</sup> The source of white light was a 260 candle-power Mazda lamp. The time of exposure was measured with a stop-watch. The exposures were also checked by means of the shutter of a photographic camera. Each starfish was kept in a rectangular glass dish during a series of experiments. The test was made by allowing the light to fall perpendicularly on the side of the dish on which the animal rested. This procedure resulted in illuminating the ventral sides of one or more rays. Withdrawal of the tube feet and beginning closure of the groove were taken as the end-point of the reaction. In case it was desired to avoid contact on the part of the tube feet, the animal had to be supported vertically in the dish while the exposure was made. The starfish must be kept in the dark for an hour before beginning the experiments and they must not be excited mechanically at the time of the test. It was found that a subliminal exposure to light preceding by a few seconds an otherwise adequate exposure, completely inhibited the reflex. Therefore only one measurement could be made at a time. Accordingly after each exposure the animals were put into freshly aerated sea water and kept in the dark for 15 minutes before being tested again.

The shortest reaction time obtainable with a light intensity of 26,000 candle-meters intensity was 1.5 seconds. The longest reaction time secured with a weak light was approximately 3 seconds. If the light intensity was so low that an exposure of more than this length of time was necessary to produce the required photochemical effect, no reaction was obtained. The minimum quantity of light

<sup>3</sup> Hecht, S., *J. Gen. Physiol.*, 1919-1920, ii, 229.



which would bring about the reaction in a dark-adapted starfish, the tube feet not being in contact with a surface, was found to have an average value of 10 to 25 candle-meter seconds.

Illumination of the dorsal surface of the starfish does not cause the retraction of the tube feet nor closure of the ambulacral groove. This shows either that the dorsal surface is insensitive to light or that nervous connection between the sensory cells of the dorsal surface and the tube foot musculature is lacking. Since, as we know from the effects of mechanical stimulation<sup>4</sup> there is nervous connection between the stereosensitive cells of the dorsal surface and the tube feet, the first hypothesis is probably correct; *i.e.*, the dorsal surface has no light receptor cells.

The "feelers" of the tips of the rays are relatively insensitive to light since they show no retraction upon illumination. Only tube feet with well developed terminal pads are strongly photosensitive. Illumination of a limited number of tube feet causes reaction only in that area, or at most, in the most sensitive individuals, only in the ray illuminated. The light reaction is therefore local in character.

It was noted in the experiments that tube feet which were not in contact with a surface retracted much more readily in response to illumination than did those which were in contact with a surface. But by the use of more intense light it was found possible to force the retraction of those in contact with a surface. This antagonism between stereotropism and the reaction due to light gives a means of quantitative treatment of stereotropism by the method of indirect measurement. It is therefore only necessary to illuminate the animal with a known quantity of light just sufficient to neutralize its stereotropism, as shown by the withdrawal of the tube feet from the surface, in order to have a measure of stereotropism in terms of light quantity. Although 10 to 25 candle-meter seconds is sufficient to cause retraction of tube feet which are simply extended in the water without touching a surface, this quantity of light has no apparent effect upon the tube feet which are in contact with the glass side of the aquarium. But if exposed to a light sufficiently powerful, starfish clinging to the side of a glass dish frequently release their hold en-

<sup>4</sup> Moore, A. R., *J. Gen. Physiol.*, 1919-20, ii, 319.

tirely and drop to the bottom. The least quantity of light which will cause the retraction of the tube feet from a surface may be regarded as the photic equivalent of stereotropism.

In making determinations of the photic equivalent of stereotropism the apparatus and procedure were the same as noted above except that the exposures were made on the ventral side of the starfish while it clung to the vertical wall of the dish. It was thus possible to measure the distance of the receptors from the light source with accuracy. Exposures were made on each animal at 5 cm. intervals (Table I). The letters in the table indicate the individual animals. The figures under the letters are the distances in centimeters at which the 260 candle-power light acting for the time interval stated in the first column will just cause the tube feet to be withdrawn from the wall and the ambulacral groove to begin to close. At a distance

TABLE I.

Exposure time.	A	B	C	D	Average $i$ .	$t \times i^*$
<i>secs.</i>						
0.5	70	60	75	60	609	305
1	85	90	110	90	304	304
2	125	130	160	140	139	278

\*  $i$  represents intensity in candle meters;  $t$ , exposure time in seconds.

of 5 cm. farther away from the light an exposure of the length designated caused no significant response. Each figure is the result of repeated trials.

While there is a considerable variation in the values when different individuals are compared, each animal yields fairly consistent results. For example, C has higher sensitivity than the others and shows the fact in each of the three exposures. The desirability of more extensive studies is apparent, but the end of the season cut short the progress of the experiments and the data given are submitted for the purpose of illustrating the possibilities of the method.

As a result of the measurements made, it can be stated that the average photic equivalent for stereotropism in *Asterias* is between 250 and 350 candle-meter seconds. This is a value 10 to 20 times as great as is required to cause retraction of the tube feet when they are

not in contact with a surface. It is also clear from the figures in the table that the length of exposure is inversely proportional to the light intensity since the product of intensity by time equals a constant. This shows that a certain quantity of light is required to bring about the reaction, which is but another way of saying that the Bunsen-Roscoe law holds here as it does in other photochemical reactions.<sup>3,5</sup>

<sup>5</sup>Loeb, J., *Forced movements, tropisms, and animal conduct*, Philadelphia and London, 1918, 83.



## COMPARATIVE STUDIES ON RESPIRATION

### XX. THE CAUSE OF PARTIAL RECOVERY.

By O. L. INMAN.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, September 29, 1921.)

New questions regarding the mechanism of injury and recovery have recently arisen. It has become apparent that, contrary to the accepted view, recovery from injury may be either partial or complete, according to circumstances. This conclusion was drawn from investigations on electrical conductivity by Osterhout<sup>1</sup> and has been confirmed by the experiments on respiration carried out by the writer.<sup>2</sup>

A question of especial interest is whether partial recovery is due to the death of certain cells or to a lowered rate of metabolism of all the cells which make up the tissue. The writer has endeavored to throw light on this question by determining the number of cells that survive after exposure to a toxic agent and by comparing the rate of respiration before and after such exposure.

The material used in these experiments was a unicellular alga, *Chlorella*. It was isolated from the soil by Dr. Schramm and grown on agar, free from bacteria and other organisms. The agar was prepared in the following manner.

Agar.....	10 gm.
Calcium nitrate.....	1.026 gm.
Magnesium sulfate (hydrated).....	0.2 gm.
Dipotassium phosphate .....	0.2 gm.
Calcium chloride (anhydrous).....	0.1 gm.
Ferrous sulfate.....	trace
Distilled water.....	1000 cc.

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<sup>1</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1920-21, iii, 145, 415, 611.

<sup>2</sup> Inman, O. L., *J. Gen. Physiol.*, 1920-21, iii, 663.

These salts were dissolved in water; the agar was then added and the whole mixture sterilized in an autoclave.

From agar the alga was transferred to a liquid medium prepared in the same manner with the exception that 10 gm. of dextrose were used in place of 10 gm. of agar.

The cultures used in these experiments were grown for a period of 30 days in this liquid medium. The medium has a very slight alkaline reaction and when tested showed no detectable buffer action.

The algæ were transferred to the respiration chamber<sup>3</sup> along with enough of the medium to make a volume of about 20 cc. The normal rate of respiration was determined by taking the time necessary for a change from pH 7.78 to pH 7.36. (This usually took about 2 minutes). When the normal rate of respiration was practically constant the algæ were separated from the medium by means of a centrifuge, which was run at a moderate speed. (Tests showed that this centrifugation caused no injury.) The reagent was then added and measurements of the rate of respiration were made at frequent intervals until the desired point below the normal rate of respiration was reached; the algæ were then returned to the normal solution and at intervals the rate of respiration was measured.

The experiments were performed at  $18 \pm 3^\circ\text{C}$ .

Fig. 1 shows the result of exposing the cells to hypertonic balanced solutions. If the respiration does not fall below 60 per cent of the normal, recovery is complete; if it falls lower recovery may be incomplete, while if the rate drops too low there is no recovery.

It was found in these experiments that there is no tendency for recovery to complete itself even when the organism is kept for days under the most favorable conditions.

Fig. 2 shows recovery after exposure to solutions of chloroform. In this, as in the previous case, no attempt was made to follow the respiration curve during the exposure since the interesting question was the amount of injury produced and the subsequent behavior. Curves *A*, *B*, and *C* show that if the injury is stopped when respiration is from 58 to 70 per cent of normal, there is in every case complete recovery within a period of about 4 hours and that the organism

<sup>3</sup> This is the apparatus described by Osterhout. Osterhout, W. J. V., *J. Gen. Physiol.*, 1918-19, i, 17.

is found at the end of 20 hours to be still normal. Where complete recovery was observed the algæ were planted upon agar plates and they grew and formed colonies in the normal manner.

Curves *G* and *F* show that there is no partial recovery but a continued state of diminished metabolism which lasts over a considerable number of hours as shown by the curves.

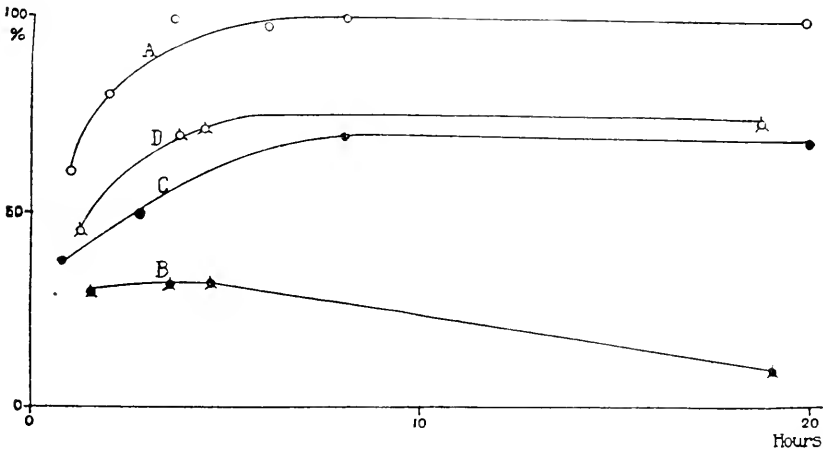


FIG. 1. Curves showing recovery of *Chlorella* from the effects of a hypertonic balanced solution of NaCl plus  $\text{CaCl}_2$ . Curve *A* shows the degree of recovery after an exposure of 60 minutes to a solution containing 50 cc. NaCl 1 M to each cc. of  $\text{CaCl}_2$  1 M; Curve *D*, after an exposure of 78 minutes to the same solution; Curve *C*, after an exposure of 48 minutes to a solution containing 50 cc. NaCl 1.25 M to each cc. of  $\text{CaCl}_2$  1.25 M; Curve *B*, after an exposure of 96 minutes to the last mentioned solution. The first point on each curve denotes the per cent to which the respiration had fallen when the algæ were transferred from the solution to the normal medium. The ordinates represent the rate of production of  $\text{CO}_2$  expressed as per cent of the normal; the abscissæ, time in hours. Each curve represents a typical experiment.

Curves *D* and *E* show that when the respiration has fallen to about 38 per cent of the normal, as the result of the exposure to chloroform, there is no recovery after removal from the chloroform, but, on the contrary, a gradual decrease in the rate.

Experiments were next undertaken to decide whether partial recovery meant that some of the cells had been killed and others had remained uninjured, or whether all the cells remained alive but had

a lowered rate of metabolism because of the treatment. In order to throw light upon this point it was decided to resort to staining methods. Methylene blue was found to be convenient since it would quickly stain the algæ that had been killed by the chloroform or the hypertonic salt solutions, but did not stain the living cells during a short exposure to the dye. In the normal culture some of the cells (from 5 to 8 per cent) were stained.

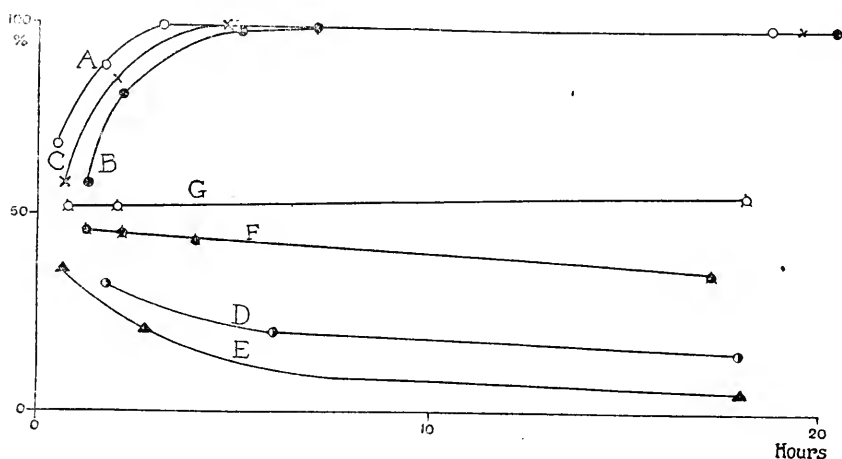


FIG. 2. Curves showing the rate of respiration of *Chlorella* after treatment with chloroform. Curves A, B, and C were obtained by using 0.225 per cent chloroform (by volume); Curves G and F with 0.27 per cent; Curve B, with 0.36 per cent. The first point on each curve denotes the per cent to which the respiration had fallen when the algæ were transferred from the solution of chloroform to the normal medium. The ordinates denote the rate of production of  $\text{CO}_2$  expressed as per cent of the normal; the abscissæ, time in hours. Each curve represents a typical experiment.

Cultures of the same age were selected and the normal respiration was measured in the usual manner. Each culture was then treated with chloroform of sufficient strength to decrease the respiration of the algæ. The algæ were then subjected to the stain (for 3 minutes) and it was found that not more than 8 per cent of the cells were ever stained,<sup>4</sup> no matter whether the respiration had fallen to 70 per cent or to 10 per cent of the normal rate. The time of exposure of the

<sup>4</sup> The cells were counted by means of a hemocytometer.



algæ to the chloroform was never more than 96 minutes. After an excessively long exposure to the chloroform (10 to 15 hours) about 95 per cent of the cells stained; in this case there is little doubt that most of the cells were killed, but such long exposures were never used in the ordinary experiments.

Staining was also tried after treatment with hypertonic salt solutions. Here partial recovery was found and yet not more than 5 to 8 per cent of the cells stained. In these experiments the longest time of exposure to the salt solution was about an hour. If the cells were treated for from 5 to 8 days with hypertonic NaCl about 95 per cent stained.

It would therefore appear that a treatment with hypertonic salt solutions which lowers the rate of respiration so much that recovery is incomplete kills few or none of the cells. If this be true then it cannot be said that partial recovery is due to the fact that some of the cells are killed by the toxic agents while others recover completely. The fact that the recovery is not complete must be due to the diminished metabolism of practically all the cells.

#### SUMMARY.

The respiration of *Chlorella* is diminished by exposure to hypertonic salt solutions. After a short exposure there is complete recovery when the algæ are removed to the normal medium. After a longer exposure recovery may be incomplete, as shown by the fact that the rate of respiration fails to rise to the normal level. Staining with methylene blue indicates that few, if any, of the cells are killed as the result of the exposure. It would therefore seem that the treatment produces a persistent lowering of the rate of metabolism. Such a condition of metabolism is also found after exposure to chloroform.



# THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE PRODUCTION OF CARBON DIOXIDE BY *BACILLUS BUTYRICUS* AND *BACILLUS SUBTILIS*.

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In connection with the study of various therapeutic agents and their effects upon the carbon dioxide production of bacteria, it was necessary to investigate the influence of changes in the hydrogen ion concentration before any conclusions could be drawn.

Clark<sup>1</sup> has given a comprehensive bibliography of the influence of hydrogen ion concentration upon various activities. Clark and Lubs<sup>2</sup> have given a summary of the limiting reactions which govern the growth of some bacteria. Most of the literature, however, is concerned with metabolic activities other than the production of carbon dioxide. Thunberg<sup>3</sup> has observed the effects of various hydrogen ion concentrations upon surviving frog muscle, and has found that there was a considerable decrease in the production of CO<sub>2</sub> in both the acid and the alkaline range.

Gustafson,<sup>4</sup> using substantially the same method as the writer, and working with *Penicillium chrysogenum*, obtained a decrease in the rate of CO<sub>2</sub> production when acid was added in quantity sufficient to make the pH 4 or less. He also found a decrease in the rate when the pH of the medium containing the organisms was 8 or more. Between these two concentrations he found no variation in the rate of CO<sub>2</sub> production.

The method used by the writer was that described in a previous publication.<sup>5</sup> The apparatus is a closed system, containing at either

<sup>1</sup> Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

<sup>2</sup> Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 222.

<sup>3</sup> Thunberg, T., *Skand. Arch. Physiol.*, 1910, xxiii, 154; 1911, xxiv, 23.

<sup>4</sup> Gustafson, F. G., *J. Gen. Physiol.*, 1919-20, ii, 617.

<sup>5</sup> Brooks, M. M., *J. Gen. Physiol.*, 1919-20, ii, 5.

end two Pyrex glass tubes, in which are placed respectively the bacteria to be used and the indicator, the change in color of which measures the respired  $\text{CO}_2$  which is forced through the system.

In order to facilitate experimentation, a non-pathogenic acid-fast organism was used. This was *Bacillus butyricus*, obtained from the stock culture of the Hygienic Laboratory, and isolated originally from butter. The rapid growth of this organism is an advantage; 48 hour cultures grown on glycerin agar and incubated at  $37^\circ\text{C}$ . were used for all the experiments. The heavy growth obtained was washed off into a 0.75 per cent solution of dextrose in distilled water.

As a basis of comparison, the same experiments were also performed with *Bacillus subtilis*, a non-acid-fast organism, which had been planted upon agar-agar and incubated for 18 hours at  $37^\circ\text{C}$ . previous to use. These organisms were then transferred to a 0.75 per cent solution of dextrose in distilled water.

For each experiment 2.5 cc. of 0.75 per cent dextrose solution containing the organisms were used. The dextrose solution had a pH of 7.0. The hydrogen ion concentration of the solution was changed by adding drops of various concentrations of NaOH or  $\text{H}_2\text{SO}_4$  from a standard dropper. In solutions requiring a greater amount of acid or alkali more concentrated solutions were used, thus keeping the volume nearly constant.

The NaOH was prepared from the best reagent obtainable, handled with all necessary precautions to avoid the absorption of  $\text{CO}_2$ , and kept under soda-lime tubes. The  $\text{H}_2\text{SO}_4$  was boiled for some time to get rid of most of the volatile impurities.

The indicators used for determining the pH were thymol blue, brom phenol blue, methyl orange, methyl red, brom cresol purple, phenolsulfonephthalein, and phenolphthalein. Buffer solutions, made according to Sørensen's tables, were used as standards for comparison.

The rate is taken as the reciprocal of the time required for the change in the pH value (in the indicator tube) from 7.8 to 7.6. This time varied according to the amount of bacterial suspension used.

The temperature was kept at  $21^\circ \pm 1^\circ\text{C}$ .

Control experiments were performed with all the solutions in the absence of bacteria, and also with dead bacteria killed by boiling for one-half hour.

Experiments were carried out for 2 hours, after which time the rate of production of  $\text{CO}_2$  remained practically constant for several hours. For this reason it was not thought necessary to include in the figures the data obtained after 2 hours. It is assumed that when the course of the reaction reached this point equilibrium was attained.

Fig. 1 shows the effect upon the rate of production of  $\text{CO}_2$  by *Bacillus butyricus* when varying amounts of  $\text{H}_2\text{SO}_4$  are added to the

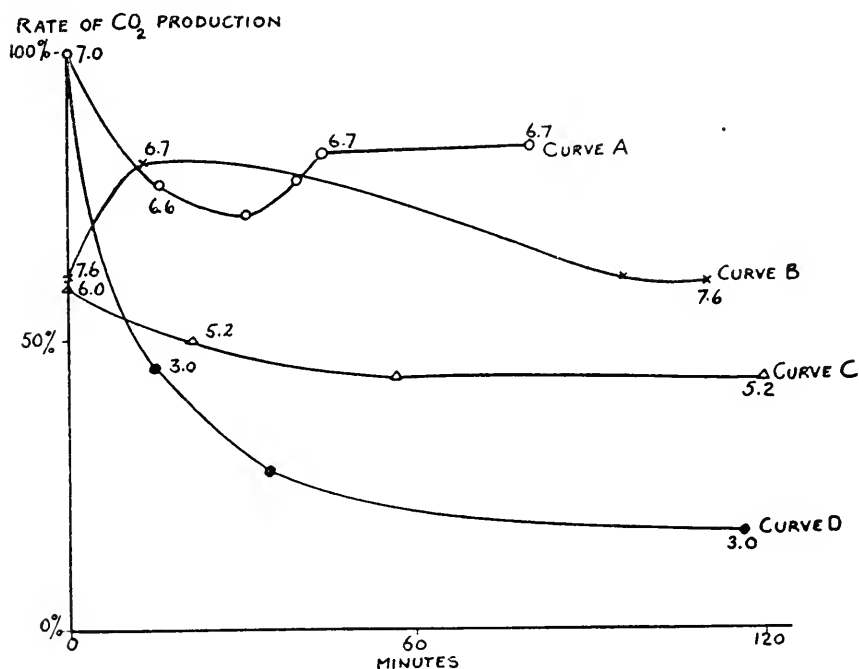


FIG. 1. Curves showing the progressive effect of acid upon the pH value and upon the rate of production of  $\text{CO}_2$  by *B. butyricus* (expressed as per cent of the normal). The point marked zero on the abscissæ indicates the initial pH value and the rate of  $\text{CO}_2$  production of the bacteria at the instant before acid is added. The figure attached to the first reading indicates the pH just after the addition of acid; subsequent figures indicate the pH values at the times when the readings were taken. The normal rate (which is taken as 100 per cent) represents a change in the pH value of the indicator tube in the apparatus from 7.8 to 7.6 in a number of seconds, depending upon the amount of bacterial suspension used, when the pH value of the organisms was 7.0. Each curve represents a single typical experiment.

medium containing the bacteria at different hydrogen ion concentrations.

It was found that the rate was at a maximum at pH 7; this was accordingly called 100 per cent, and arbitrarily designated as the "normal" rate. All rates were then designated as per cent of the normal. When, for example, the initial pH was 6, enough alkali was added to make the pH 7. The rate at pH 6 was then divided by the rate at pH 7 in order to express the rate at pH 6 as per cent of the normal rate.

The initial pH value of the dextrose containing the bacteria was not always the same owing to variations in the organisms themselves. It was found impracticable to adjust the pH at the beginning of each experiment to a value which would be uniform for all experiments (for example to pH 7.0). For this reason the curves in Fig. 1 and Fig. 2 do not all begin at 100 per cent, but at different rates of production of  $\text{CO}_2$  depending on the initial pH value of the dextrose containing the organisms.

In Fig. 1 and Fig. 2 the acid or alkali respectively were added at the zero point on the abscissæ. The first reading in each case denotes the rate of production of  $\text{CO}_2$  at the instant before the acid or alkali was added, and the pH at this instant is shown by the attached figure. The addition of acid or alkali changed the pH to the value indicated by the figure attached to the first reading. Thus in curve *B*, Fig. 1, the pH at the start was 7.6 and the addition of acid changed it to 6.7, as shown by the figure attached to the first point on the curve.

Curve *A*, in Fig. 1, shows the effects of the addition of acid to bacteria contained in a solution whose initial pH was 7.0. The pH fell at once to 6.6 then rose to 6.7. The production of  $\text{CO}_2$  fell and rose with the change in pH value.

Curves *C* and *D* illustrate the effect of adding larger amounts of acid: in these cases the pH remains stationary after the addition of acid while the production of  $\text{CO}_2$  falls steadily until equilibrium is attained.

Curve *B* illustrates the effect of adding acid to an alkaline suspension of bacteria (pH 7.6). The pH changed at once to 6.7 and subsequently fell. The production of  $\text{CO}_2$  rose and fell in a characteristic manner.

Fig. 2 shows the effects upon the rate of production of  $\text{CO}_2$  by *Bacillus butyricus* when varying amounts of NaOH are added to the medium containing the bacteria at various H ion concentrations.

Curve A shows the effect upon the rate of production of  $\text{CO}_2$  when to a bacterial suspension whose pH is 7.0, sufficient NaOH is added to make the pH 8.2. There is a considerable decrease in the rate which becomes 21 per cent of the normal. This is followed by an increase in the rate as the hydrogen ion concentration returns to neutrality.

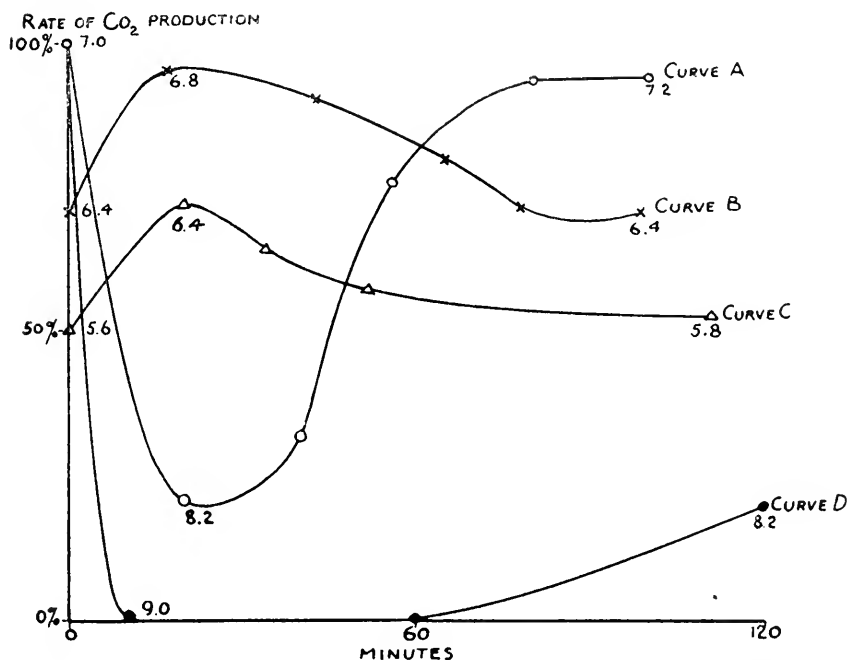


FIG. 2. Curves showing the progressive effect of alkali upon the pH value and upon the rate of production of  $\text{CO}_2$  by *B. butyricus* (expressed as per cent of the normal). The point marked zero on the abscissæ indicates the initial pH value and the rate of  $\text{CO}_2$  production of the bacteria at the instant before the NaOH was added. The figure attached to the first reading indicates the pH just after the addition of acid; subsequent figures indicate the pH values at the times when the readings were taken. The normal rate (which is taken as 100 per cent) represents a change in the pH value of the indicator tube in the apparatus from 7.8 to 7.6 in a number of seconds, depending upon the amount of bacterial suspension used, when the pH value of the organisms was 7.0. Each curve represents a single typical experiment.

Curve *B* shows the effect when sufficient NaOH is added to change the reaction from pH 6.3 to pH 6.8. There is an increase in the rate as the hydrogen ion concentration approaches neutrality; this is followed by a decrease. At the same time there is an increase in the hydrogen ion concentration. The final pH value in this case coincides with the initial pH value.

Curve *C* shows the effect when sufficient alkali is added to change the pH value from 5.6 to 6.4. There is an increase in the rate followed by a decrease, and the pH value becomes gradually less.

Curve *D* shows the effect when the pH value is changed from 7.0 to 9.0. There is a complete cessation in the rate for several hours; this is followed by a gradual increase in the rate as the medium becomes more acid. The increasing acidity is not attributable to the carbon dioxide produced, as this was frequently removed during the period of experimentation.

Briefly summarized, when alkali is added to solutions containing *Bacillus butyricus* of various hydrogen ion concentrations, the rate of production of CO<sub>2</sub> increases as the normal rate (at pH 7.0) is approached and decreases in the opposite direction.

The experiments performed with *Bacillus subtilis* gave results similar to those expressed in Fig. 1 and Fig. 2 (*Bacillus butyricus*).

Both organisms show a tendency to resist changes in the pH value. When acid or alkali is added there is always an initial effect which is quite pronounced, increasing or decreasing the pH value. This result is followed by a return towards the initial pH value. This might be due to absorption of the added acid or alkali by the organism or to the excretion of a neutralizing substance. In the case of change in the rate of CO<sub>2</sub> production produced by alkali the time required for recovery is considerable, but the organisms seem to be able gradually to overcome the cause of the depression in the rate.

Fig. 3 represents composite curves of a number of experiments with *Bacillus butyricus* (curve *A*) and *Bacillus subtilis* (curve *B*). The abscissæ are the various pH values, and the ordinates are the corresponding rates of CO<sub>2</sub> production. Each point represents the average of a number of experiments. In most cases the rate was obtained after the bacteria had been in contact with the acid or alkali from 30 to 45 minutes and had reached equilibrium (constant rate of CO<sub>2</sub> production).



A comparison of the two curves shows that the rate of production of  $\text{CO}_2$  by both organisms is a function of the pH value of the medium. Below pH 5.0 *Bacillus subtilis* is very sensitive to the effects of acid, while *Bacillus butyricus* is only gradually affected as the concentration of the hydrogen ions increases. It is not possible to state, on

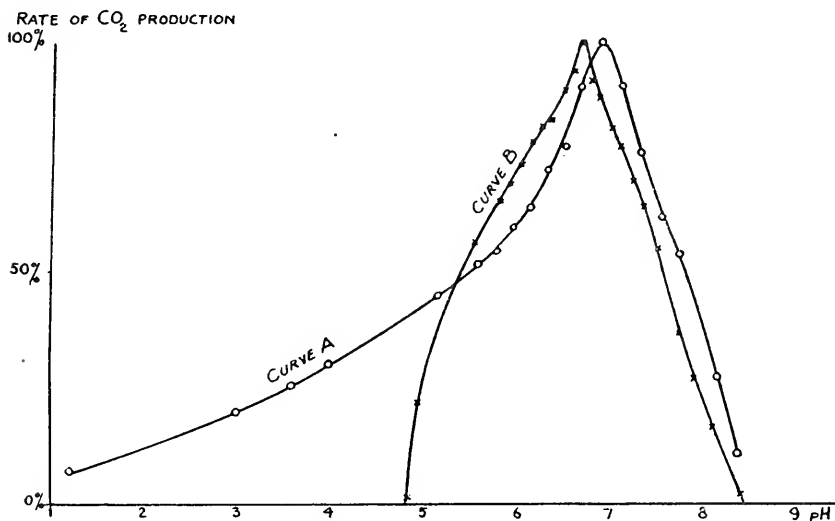


FIG. 3. Rate of production of  $\text{CO}_2$  (expressed as per cent of the normal) *B. butyricus*, Curve A, and of *B. subtilis*, Curve B, as affected by the hydrogen ion concentrations.

The normal rate represents the maximum production of  $\text{CO}_2$ . This was obtained at pH 7.0 in the case of *B. butyricus* and at pH 6.8 in the case of *B. subtilis*. It was measured by noting the number of seconds required to change the pH value of the indicator tube from 7.8 to 7.6. The time required depended upon the amount of bacterial suspension used. Each point on the curve represents an average of several experiments. Probable error of the mean less than 2 per cent except in the last two points of the alkaline range, where it is less than 3 per cent.

the basis of these experiments, whether or not this is due, as some investigators believe, to existence of a waxy envelope in the case of the acid-fast bacteria, and to the absence of such a covering in the case of the non-acid fast bacteria.

It was found that when a small amount of acid was added to suspensions of either *Bacillus butyricus* or *Bacillus subtilis* the addition

of an equivalent amount of NaOH to the medium produced complete recovery, as shown by the production of  $\text{CO}_2$ . No recovery was obtained by washing, centrifugalizing, and rewashing the organisms in dextrose solution without the addition of NaOH. When a greater amount of acid was added, the addition of NaOH produced only partial recovery. When the medium was made extremely acid, no recovery took place.

Recovery from the effects of the addition of alkali to these organisms was spontaneous, varying in the time required for recovery according to the amount of alkali added. When an equivalent amount of acid was added recovery was hastened.

In short, decreases in the rate of  $\text{CO}_2$  production caused by the addition of acid to these organisms were irreversible or only partially reversible (except in certain cases in which slight additions of acid were neutralized by a subsequent addition of alkali) while decreases of similar magnitude caused by the addition of alkali, were spontaneously reversible. These results are essentially similar to those obtained by Gustafson<sup>4</sup> in his recovery experiments with *Penicillium*.

It is evident that the effects of NaOH are not to be ascribed, to any measurable extent, to the action of Na, since previous experiments<sup>5</sup> have shown that when NaCl is added to *Bacillus subtilis* there is a decrease in the  $\text{CO}_2$  production only when the total concentration of NaCl is greater than 0.15 M. In all of the above experiments the concentration of NaOH used was considerably less than this (about one-tenth as great).

It may be objected that the observed decrease in the carbon dioxide production caused by the addition of NaOH is in reality due to buffer action or to the formation of carbonates and bicarbonates as a result of the neutralization by NaOH of the  $\text{CO}_2$  produced by the bacteria. In view of the fact that the greatest amount of alkali added in any experiment was one drop of 0.1 N NaOH it did not seem probable that this buffer action was important. The following series of experiments shows that it is negligible. The normal rate of production of  $\text{CO}_2$  by 2 cc. of an emulsion of living bacteria was determined. A tube containing 2 cc. of distilled water was then inserted into the system so that the  $\text{CO}_2$  coming from the bacteria

would pass through the distilled water before going into the indicator tube. After the rate of production of  $\text{CO}_2$  under these conditions had been determined, a drop of  $\text{NaOH}$  was added to this inserted tube and its effect upon the apparent rate of  $\text{CO}_2$  production, independent of any effect upon the bacteria, was measured. Equilibrium is very rapidly attained, since there is very little  $\text{NaOH}$  present in proportion to the amount of  $\text{CO}_2$  produced, and since the method of stirring is very efficient. The  $\text{NaOH}$  in this tube was then replaced by distilled water and a drop of the alkali of the same concentration was placed in the tube containing the bacteria. The difference between the results in the two cases would measure the action of the  $\text{NaOH}$  upon the organisms themselves. (When this experiment was varied by using 0.75 per cent dextrose in place of distilled water the results were essentially the same.)

It was found that the addition of one drop of 0.1  $\text{N}$   $\text{NaOH}$  had no measurable buffer effect. This was also the case when the alkali was added to 2 cc. of an emulsion of dead bacteria in the side tube instead of to distilled water. The addition of alkali to the dead bacteria would of course simulate more nearly the buffer effects of the actual experiments.

It is not proposed to discuss at length in this paper the theoretical basis for the reactions governing the distribution of  $\text{CO}_2$  in the apparatus. Equilibrium in the different carbonate-bicarbonate systems seems to be established almost simultaneously. Regular readings may be obtained as quickly as 5 minutes after the introduction of a solution. The hydrogen ion concentrations in the three tubes (bacterial suspension, side tube, and indicator tube) differ even when in equilibrium with the same  $\text{CO}_2$  tension because the amount of base in the three tubes differs. They may be supposed to be in equilibrium at the beginning of a reading. After this, as the  $\text{CO}_2$  is produced by the organisms there is an increase of  $\text{CO}_2$  tension in the circulating air from which  $\text{CO}_2$  is absorbed by the solutions in the tubes. The indicator tube being farthest from the source of  $\text{CO}_2$  naturally may be expected to lag very slightly behind the side-tube in its change toward equilibrium, because the latter absorbs part of the  $\text{CO}_2$ , thus lowering the  $\text{CO}_2$  pressure in the air which passes through it. In these experiments the side tube contained only 2 cc. of liquid, which had no measurable effect.

It appears therefore that the observed diminution of carbon dioxide production caused by the addition of NaOH to bacterial emulsions cannot be the result of any so called buffer action, but it is a true expression of the effect of an alkaline environment upon the bacteria.

#### SUMMARY.

1. The maximum rate of CO<sub>2</sub> production of *Bacillus butyricus* was found to be at a pH value of 7; of *Bacillus subtilis* at pH 6.8. If the pH value be raised or lowered there is a progressive decrease in the rate of production of CO<sub>2</sub>.

2. Spontaneous recovery follows the addition of alkali to either organism, while addition of acid is followed by recovery only upon addition of an equivalent amount of alkali, and is not complete except when the amount of acid is very small.

# THE INFLUENCE OF ELECTROLYTES ON THE SOLUTION AND PRECIPITATION OF CASEIN AND GELATIN.

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## I. INTRODUCTION.

The modern concepts of colloid chemistry originated largely from a study of the precipitation of colloids by electrolytes, and this accounts, perhaps, for the fact that the tendency to form aggregates was considered the chief characteristic of colloids in solution. As a consequence it is assumed in the text-books of colloid chemistry that the ultimate units of a colloid in solution are not isolated molecules or ions but larger aggregates of molecules or ions, the so called micella (small crumbs) of Naegeli, which are supposed to be kept in a stable solution or suspension through forces of repulsion due to the fact that they are electrically charged. The precipitation of the particles by electrolytes is ascribed to a diminution of the charge of the micella through the adsorption of the ions of the electrolyte. It was noticed by Hardy that the active or precipitating ion of the electrolyte was always that ion which had the opposite sign of charge as the colloidal particle, and Picton and Linder had noticed that the precipitating efficiency of ions increased rapidly with their valency.<sup>1</sup>

The experiments published by one of us have led to results which are compatible with some but not all of the assumptions just enumerated. In the first place, experiments on the osmotic pressure and the viscosity of protein solutions have made it probable that the ultimate units in certain protein solutions, such as crystalline egg albumin, are essentially isolated protein ions or molecules, though such solutions may, in a secondary way, also contain aggregates of ions or molecules.<sup>2</sup>

<sup>1</sup> Zsigmondy, R., *Kolloidchemie*, Leipsic, 2nd ed., 1918.

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 827; 1921-22, iv, 73, 97.

It was, secondly, found that proteins combine with acids and bases according to the stoichiometrical laws of classical chemistry, and that there is neither any need nor, in fact, any room for the assumption that the ions of electrolytes are adsorbed by the protein particles in solution.<sup>3</sup> Thirdly, the electrical charges of protein particles which occur in such solutions are due chiefly, if not exclusively, to either the electrolytic dissociation of protein salts or to membrane potentials caused by the Donnan equilibrium.<sup>4</sup> Fourthly, the influence of electrolytes on the p.d., the osmotic pressure, and the viscosity of protein solutions<sup>5</sup> and the swelling of gels<sup>6</sup> can be explained quantitatively on the basis of the Donnan equilibrium. Under these circumstances it became necessary to find out whether or not the influence of electrolytes on the precipitation of proteins can be harmonized with these recent results.

All the workers who have studied the influence of electrolytes on the precipitation of colloids have come to the conclusion that there are two distinct groups of phenomena. In the one group the precipitation requires high concentrations of an electrolyte, while in the second, a low concentration of electrolyte suffices for precipitation. The difference between these two cases is so striking that it has been used for a classification of colloids. Thus, according to Zsigmondy, Noyes<sup>7</sup> discriminates between "colloidal solutions" and "colloidal suspensions;" the "colloidal solutions" being characterized by a high degree of viscosity, by a tendency to gelatinize, and by the fact that they are not easily precipitable by electrolytes; while the "colloidal suspensions" are, according to Noyes, characterized by a low order of viscosity, by a lack of tendency to gelatinize, and by the fact that they are easily precipitated by electrolytes.<sup>8</sup>

<sup>3</sup> Loeb, J., *Science*, 1920, lii, 449.

<sup>4</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 667.

<sup>5</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 557, 667, 691, 827; 1921-22, iv, 73, 97.

<sup>6</sup> Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

<sup>7</sup> Noyes, A. A., quoted in Zsigmondy, R., *Kolloidchemie*, Leipsic, 2nd ed., 1918, 29.

<sup>8</sup> Different authors have introduced different terms for these two groups of colloids, Perrin speaks of hydrophilic and hydrophobic colloids; Freundlich of lyophilic and lyophobic, and Wo. Ostwald of emulsoids and suspensoids.

Of all the characteristics for the two groups mentioned by Noyes, only one is essential, namely, the difference in the ease with which the two groups are precipitated by electrolytes, while the two other differences mentioned by him are only accidental. Thus a solution of crystalline egg albumin requires at ordinary temperature a high concentration of electrolyte for precipitation, although the viscosity of such a solution is low and although the solution has no tendency to gelatinize; while at a sufficiently high temperature, low concentrations of electrolytes will precipitate crystalline egg albumin though the viscosity of the solution is now high and though the solution has now a tendency to gelatinize. Hence only one of the differences mentioned in Noyes' definition remains; namely, the difference in the relative concentration required to precipitate colloids from their solution or their suspension.

The discrimination between the two types of precipitation according to whether high or low concentrations of electrolytes are required, is, however, essential and it becomes our first problem to account for this difference. We shall see that it is accompanied by another difference. When high concentrations of electrolytes are required for the precipitation of proteins the sign of charge of the protein particles is of little significance. Sulfates are much more effective for the salting out of gelatin or crystalline egg albumin from their watery solution than chlorides, regardless of whether the solution of the protein is at the isoelectric point or on the acid or alkaline side of it.

When low concentrations suffice for precipitation, the sign of charge of the protein ions becomes of paramount importance. On the acid side of the isoelectric point the active ion of the precipitating salt is the anion, while on the alkaline side it is the cation. It happens that this is also true for the Donnan effect and that in the Donnan effect also, low concentrations of electrolytes suffice for the depressing effect of a salt. This suggests the possibility that precipitation of the second group of colloids, *i.e.*, where low concentrations are required, is in some way connected with the Donnan equilibrium; and that Hardy's rule is only the consequence of this fact; while where high concentrations of electrolytes are required for precipitation the forces determining the process have no connection with the Donnan equilibrium.

In order to gain more definite information concerning the nature of the forces involved in the two cases it seemed advisable to supplement these investigations by a study of the mechanism of the solution of proteins. We have studied the mechanism of solution of granules of isoelectric casein in acid and in alkali and find that this mechanism is entirely different in the two cases. The solution of casein chloride is controlled by forces connected with the Donnan equilibrium; and it also happens that casein chloride when in solution can be precipitated by low concentrations of electrolytes; *e.g.*,  $M/8$  NaCl. On the other hand, the solution of sodium caseinate is apparently controlled by the forces of chemical attraction between water and certain groups of the casein molecule; and it happens that very high concentrations of electrolytes, *e.g.*,  $2\frac{1}{2}$   $M$  NaCl or  $3\frac{1}{4}$   $M$  LiCl, may not be sufficient for precipitation.

## *II. The Precipitation and Solution of Casein Chloride.*

1 per cent solutions of casein chloride of pH 2.2 were prepared in different concentrations of salts in water of about the same pH. That concentration was determined which causes an almost instantaneous complete precipitation of the protein from the originally milky solution so that the supernatant liquid became as clear as water. These concentrations were as follows:

NaCl .....	about $M/8$
NaNO <sub>3</sub> .....	about $M/8$
CaCl <sub>2</sub> .....	about $M/8$
Na trichloracetate .....	about $M/16$
Na <sub>2</sub> SO <sub>4</sub> .....	about $M/32$

Though the results are only semiquantitative, the validity of Hardy's rule and the valency effect are easily recognizable. Beside these two effects, some constitutional effects of the anion (such as the trichloracetate) may exist. It is also obvious that the concentrations of electrolytes required for instantaneous, complete precipitation of casein chloride are considerably lower than those required for the precipitation of Na caseinate from their watery solution, so that we can be sure that in the case of casein chloride we are dealing with a representative of the "suspension" group of colloids, in the sense of Noyes.



It can be shown that the solution of the casein chloride depends on forces regulated by the Donnan equilibrium and that the rule of Hardy is in this case at least only a consequence of this fact. This can be proven by microscopic observation of the mechanism of the solution of solid particles of originally isoelectric casein in solutions of acids of different concentration. It was found that the particles of casein swell in a solution of HCl, becoming more and more transparent the more they swell, and that when the swelling has reached a certain stage, the particles disappear—they are dissolved. When in the swollen stage, slight agitation may make them fall apart. T. B. Robertson had suggested such a mechanism for the solution of Na caseinate,<sup>9</sup> but it was found that the mechanism of solution in this latter case is different. There is no doubt, however, that the swelling of casein particles is a necessary prerequisite for the solution of casein-acid salts, since such particles are only dissolved when their swelling exceeds a definite limit.

The method of procedure was as follows: A small number of granules of isoelectric casein of the same size (going through a sieve with mesh 100 but not through a sieve with mesh 120) were put into 50 cc. of water containing different quantities of different acids and kept at 24°C. In various intervals, *i.e.*, after 15, and 60 minutes, and 6, and 24 hours, the diameter of about 15 grains was measured with a micrometer in a microscope and the average diameter calculated. The particles were not stirred, and care was taken to avoid their breaking into smaller fragments. The averages after 1 hour are plotted in Fig. 1. The abscissæ are the logarithms of the concentrations of acid of the watery solution, the ordinates are the average diameters of the particles. It is obvious that the average diameter of the particles increases at first with the increase of the concentration of the acid, reaching a maximum at about pH 2.0 of the outside solution, and with a further increase in the concentration of the acid the swelling becomes less again.

Fig. 2 gives the measurements of the same particles after 24 hours. At this time all the particles in the region of greatest solubility for HCl and for H<sub>3</sub>PO<sub>4</sub>, *i.e.*, between pH of the outside solution of 1.8 and 2.9, had completely dissolved and could no longer be measured.

<sup>9</sup> Robertson, T. B., *The physical chemistry of the proteins*, London, Bombay, Calcutta, and Madras, 1918, 275 ff.

Figs. 1 and 2 show another fact; namely, that the rate of swelling is not the same in different acids. It is about the same in  $\text{HCl}$  and  $\text{H}_3\text{PO}_4$  (for the same pH) but decidedly less in  $\text{HNO}_3$  and still less in  $\text{H}_2\text{SO}_4$  and trichloroacetic acid. It was found that the rate of solution

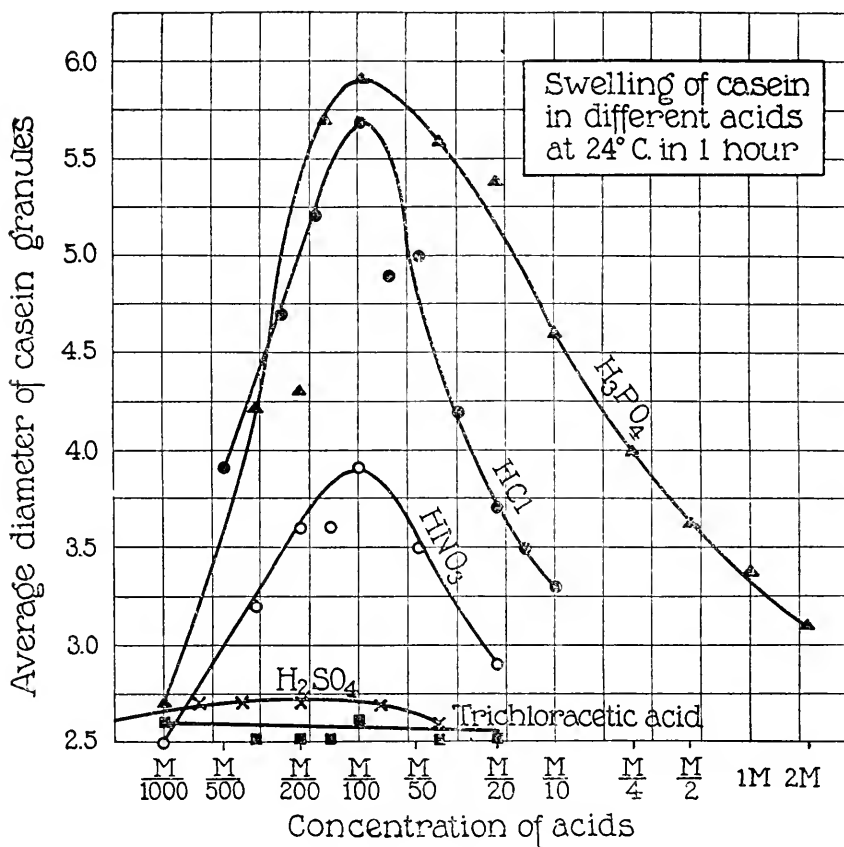


FIG. 1. Relative swelling of isoelectric granules of casein when put into acids of different concentrations, at 24°, after 1 hour. The hydrogen ion concentration of the casein particles is less than that of the outside solution on account of the Donnan equilibrium. The latter concentrations are plotted as abscissæ. The ordinates are the relative average diameters of the granules.

of casein in these different acids followed closely the rate of swelling. It took longer to dissolve casein in  $\text{HNO}_3$  than it did in  $\text{HCl}$  (at 20°C.); and the casein was practically insoluble in  $\text{H}_2\text{SO}_4$  and trichloroacetic acid in 24 hours.

The rate of swelling is a function apparently not only of the Donnan equilibrium, but also of the force of cohesion between the particles. Procter and Wilson<sup>6</sup> have suggested that the rapid increase of swelling of solid gelatin with a rise in temperature is due to a corresponding

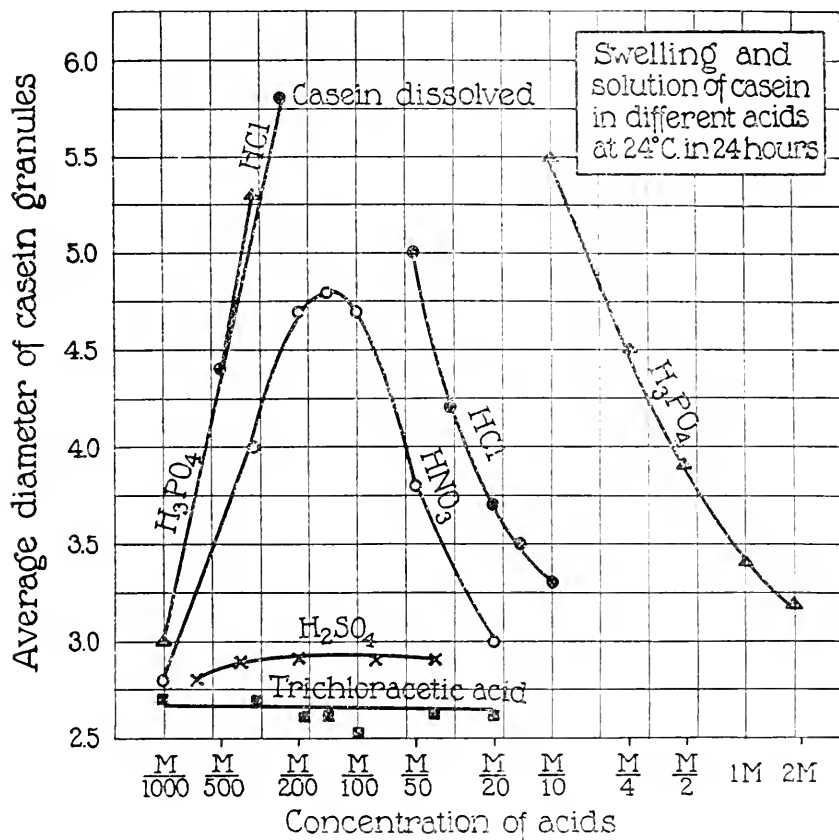


FIG. 2. Swelling of the same granules after 24 hours. Where the swelling exceeds a certain limit, between 5 and 6 in our scale, the particles are dissolved, thus showing that swelling is of importance in the mechanism of solution of casein chloride.

diminution of cohesion between the molecules of gelatin with rising temperature. The influence of the anion of gelatin-acid salts on the cohesion of the particles of a solid gel is apparently much less than the influence of the anion on the cohesion of casein particles. The

forces of cohesion in the case of casein sulfate and casein trichloracetate seem to be so great that they cannot be overcome by the osmotic pressure due to the Donnan equilibrium; and hence no swelling (and as a consequence no solution) of solid casein is possible in  $\text{H}_2\text{SO}_4$  or trichloroacetic acid.

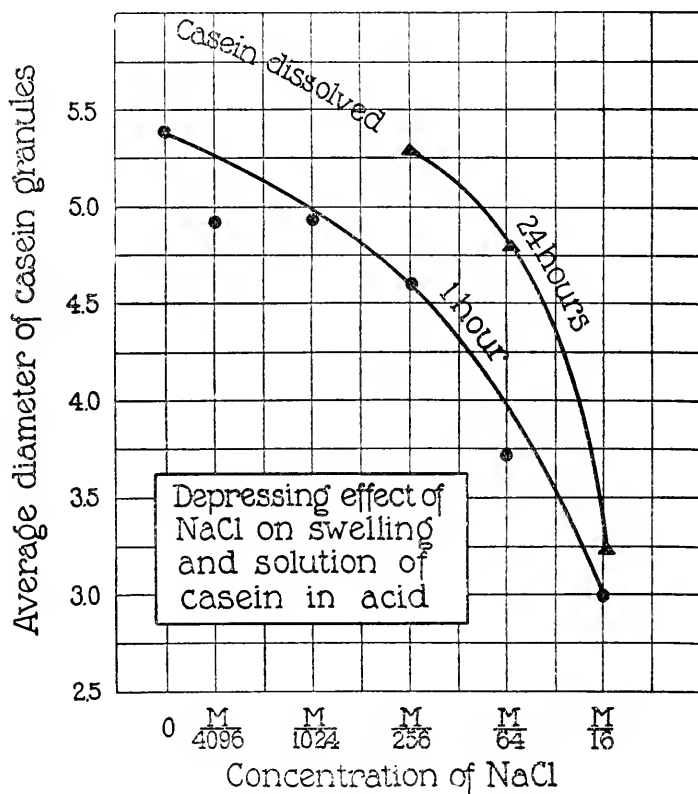


FIG. 3. Depression of swelling of casein particles in  $\text{M}/100$   $\text{HCl}$  by the addition of  $\text{NaCl}$ . A low concentration of acid suffices already for a noticeable depression of the swelling. The lower curve represents swelling after 1 hour; the upper curve swelling after 24 hours. Solution of the swollen particles occurs when the average diameter of the particles exceeds the value 5 to 6 in our scale.

Procter and Wilson have shown that the theory of the Donnan equilibrium explains the depressing effect of a salt on the swelling of solid gelatin.<sup>6</sup> Microscopic measurements of the influence of  $\text{NaCl}$  on the rate of swelling of individual grains of casein particles in  $\text{M}/100$   $\text{HCl}$  were made at  $24^\circ\text{C}$ . and the results plotted in Fig. 3.

The ordinates are the average diameters of the particles after 1 and 24 hours respectively. The abscissæ are the concentrations of NaCl. The depressing effect is similar to that found in the case of the swelling of a jelly of gelatin. After 24 hours the particles had dissolved in the NaCl solutions of a concentration of below  $m/256$ , but not in concentrations of NaCl higher than  $m/256$ .

That the solution of casein chloride is thus regulated to a large extent by the Donnan effect was ascertained also by measurements of the quantity of casein chloride dissolved at 20°C. at various pH of the solution. 1 gm. of isoelectric powdered casein was put into 100 cc. of solutions of HCl of different concentration and kept in these solutions in one case for 1 hour, in a second case for 22 hours. The mass was then poured into graduated cylinders and the undissolved part was allowed to settle to the bottom for 2 and for 6 hours respec-

TABLE I.

*Amount of Casein Dissolved at 20°C in HCl of Different pH.*

pH	4.36	3.32	3.11	2.97	2.94	2.84	2.75	2.64	2.53	2.36	2.18	2.06	1.87	1.66	1.50	1.40
Mg. dissolved after 1 hour.....	42	55	86	249	265			348	408		547	538	401	366	272	219
Mg. dissolved after 22 hours.....	102	133	164	267	342	459	536	634	646	733	788	779	710	528	374	300

tively at 20°C. The supernatant liquid was removed and the sediment dried over night in an oven at about 100°C. Table I gives the result. The dry weight of 1 gm. of isoelectric casein was found to be 0.870 gm. and this weight diminished by the dry weight of the sediment was the amount dissolved. Table I shows that, the rate of solution increases with diminishing pH from 4.4 to 2.18 where the solubility of casein chloride is a maximum; with a further decline in pH the solubility diminishes again. This is in agreement with the Donnan effect.

In a similar way the depressing effect of NaCl on the rate of solution of casein chloride was ascertained. Solutions of 12.5 cc. of 0.1  $N$  HCl in 100 cc. and containing 1 gm. of powdered, originally isoelectric casein were prepared in 0,  $m/2048$ ,  $m/1024$ , to  $m/4$  NaCl. The pH of a solution of 1 gm. casein in 100 cc. containing 12.5 cc. of 0.1  $N$  HCl was 2.12 and this pH was the same in all solutions made up in NaCl.

The solution was kept at 20° for 16 hours and then allowed to settle for 24 hours at 20° in 100 cc. graduate cylinders. The dry weight of the sediment was determined and this weight when deducted from the dry weight of 1 gm. isoelectric casein, namely, 0.870 gm., was the amount that had gone into solution after a correction was made for the free NaCl held in 2 cc. solution which was arbitrarily assumed not to have been removed. Though this latter correction was somewhat arbitrary, it could have caused a noticeable error only when the concentration of the salt solution exceeded M/64. For the solutions of M/64 and below this error was negligible. Table II gives the number of milligrams of casein which had gone into solution.

TABLE II.

	Concentration of NaCl.					
	M/2048	M/1024	M/512	M/256	M/128	M/64
Mg. dissolved.....	714	685	665	615	449	282

The main fact is that a slight increase in the concentration of NaCl causes already a noticeable drop in the rate of solution. Thus M/1024 NaCl causes already a noticeable diminution in the solubility of a 1 per cent solution of casein chloride of pH 2.12 at 24°C.

These observations then indicate that the solution of solid particles of casein chloride is brought about by the ultimate elements being forced apart mechanically through the process of swelling. The force acting in this swelling is the hydrostatic pressure of the water which is forced into the interstices of the solid particles by the osmotic pressure in the interstices between the casein ions. Procter and Wilson have shown that the application of Donnan's theory of membrane equilibrium accounts quantitatively for this swelling on the assumption that swelling is caused by the excess of the osmotic pressure inside the gel over that of the surrounding solution. As soon as the osmotic pressure in the gel exceeds the forces of cohesion between the casein ions of the gel the casein ions constituting the gel are separated.

The question then arises: How can the Donnan effect stabilize the particles of casein chloride in solution, and how can we explain the

precipitating effect of low concentrations of neutral salts? Let us assume that the ultimate particles in a solution of casein chloride of pH 2.2 are, (a) isolated casein ions, (b) isolated casein molecules, and (c) small casein aggregates or micella. The Donnan equilibrium furnishes two kinds of forces preventing that degree of coalescence of these particles which is required for precipitation; namely, the osmotic pressure and the membrane potentials. When isolated protein ions collide and remain attached to form a micellum, a Donnan equilibrium is established between the nascent micellum and the surrounding solution. The Donnan equilibrium demands that there be a higher concentration of electrolytes inside than outside and this difference in osmotic pressure leads to water being attracted into the micellum. The increase in hydrostatic pressure will force the protein molecules apart again and thus tends to prevent the formation of the micellum. Moreover, if micella exist in the casein chloride solution (aside from isolated casein ions and molecules) the coalescence of different micella into larger aggregates must be prevented by the potential difference between the micella and the surrounding solution. J. A. Wilson suggested in 1916<sup>10</sup> that the source of the charges might be the Donnan equilibrium, and one of us has recently shown that these potential differences between a gel and the surrounding solution, which Donnan's theory demands and which Wilson postulated, actually exist.<sup>11</sup> As a consequence of this P.D., the micella are charged and must repel each other according to the charge. This charge caused by the Donnan equilibrium is a minimum at the isoelectric point, rises with increasing hydrogen ion concentration, reaching a maximum, and diminishes again with a further increase in hydrogen ion concentration as shown in a preceding paper. The osmotic pressure and charge are also diminished by the addition of salt.

Hardy's rule that only that ion of a neutral salt is active in precipitation which has the opposite sign of charge as the colloidal ion, and that the efficiency of the ion increases with the valency is simply the expression of the Donnan effect, as is also the fact that very low concentrations of electrolytes suffice for precipitation. The reader will notice that it is unnecessary to assume that the ions

<sup>10</sup> Wilson, J. A., *J. Am. Chem. Soc.*, 1916, xxxviii, 1982.

<sup>11</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 557, 667.

are adsorbed by the casein and that this adsorption of ions annihilates the electrical charges on the particles of casein.

Emulsions of oil drops in water are prevented from coalescing by their electrical charges (and not by osmotic forces). The investigations of Beutner<sup>12</sup> have shown that the P.D. at the boundary of water and substances immiscible in water is determined by an unequal distribution of crystalloidal ions between the two phases, and the writer ventures to suggest that this distribution may be regulated by Donnan's theory, owing to the fact that, *e.g.*, the oleic acid anion in oil cannot diffuse into the water. The similarity of the behavior of the P.D. between oil and water and between solid gelatin and water is rather striking. The depression of the charge on the particles through the addition of salt can in both cases be explained without the adsorption hypothesis.

### *III. The Precipitation and Solution of Na Caseinate.*

When we prepare solutions of Na caseinate of pH 11.0 containing 1 gm. of originally isoelectric casein in 100 cc., we notice that it requires enormous concentrations of NaCl or LiCl to precipitate the casein. NaCl as concentrated as  $2\frac{1}{2}$  M and LiCl as concentrated as  $3\frac{1}{4}$  M were not able to precipitate Na caseinate from its solution, while M/8 NaCl was sufficient for instantaneous and complete precipitation of casein chloride from its solution. Hence Na caseinate belongs to the other group of colloidal solutions; namely, those which require high concentrations of electrolytes.

When a grain of isoelectric casein is put into a solution of NaOH Na caseinate is formed at the surface of the solid granule. This dissolves in water, as any crystalloidal substance does (*i.e.*, by the forces of residual valency), except for the accidental fact that the solution of Na caseinate seems to lower the surface tension of the watery solution at the interface, as is suggested by the rather violent spreading of the Na caseinate solution at the interface, which can be observed under the microscope. As a result projecting particles at the surface of the granule are torn away from the surface of the solid casein, collect-

<sup>12</sup> Beutner, R., *Die Entstehung elektrischer Ströme in lebenden Geweben*, Stuttgart, 1920.



ing at a short distance, while the originally rough surface of the grain of casein becomes smooth. These phenomena of solution accompanied by spreading occur soon all over the surface. The small particles of casein carried away in the spreading motion are gradually completely dissolved and the result is a solution of Na caseinate of a high osmotic pressure, indicating that the solution consists to a large extent of isolated protein ions.

The mechanism of the solution of casein in NaOH is essentially that of the solution of a crystal except for the purely accidental and secondary phenomenon that the surface tension of the Na caseinate solution is considerably lower than that of a watery NaOH solution, and that thus the orderly process of solution noticeable in the case of crystals is disturbed by phenomena of spreading. The mechanism of solution of grains of solid casein in NaOH resembles the solution of sodium oleate in water, which is also accompanied by a lowering of the surface tension at the interface. This interference of the phenomena of spreading in the process of solution of Na caseinate may account for the fact that Robertson<sup>9</sup> was unable to confirm in this case the solution theory of Noyes and Whitney,<sup>13</sup> according to which there exists at the boundary of crystals and water a film of solution which is always saturated, the velocity of solution being determined by the rate of diffusion of the dissolved crystalloid out of this film into the body of the fluid. It is natural that this theory should be masked whenever the process of solution is accompanied by phenomena of spreading due to a lowering of surface tension at the interface. The forces dragging the particles of Na caseinate into solution are to all appearances those forces of residual valency between certain groups of the molecule of Na caseinate and water which also cause the solution of crystalloids in water, while the forces dragging the particles of casein chloride into water are forces which have their origin in the Donnan equilibrium. A quotation from Langmuir will illustrate the rôle which active groups of a complex molecule may play in the process of true solution.

"Acetic acid is readily soluble in water because the COOH group has a strong secondary valency by which it combines with water. Oleic acid is not soluble because the affinity of the hydrocarbon chains for water is less than their affinity

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<sup>13</sup> Noyes, A. A., and Whitney, W. R., *Z. physik. Chem.*, 1897, xxiii, 689.

for each other. When oleic acid is placed on water the acid spreads upon the water because by so doing *the COOH can dissolve in the water* without separating the hydrocarbon chains from each other.

"When the surface on which the acid spreads is sufficiently large the double bond in the hydrocarbon chain is also drawn onto the water surface, so that the area occupied is much greater than in the case of the saturated fatty acids.

"Oils which do not contain active groups, as for example pure paraffin oil, do not spread upon the surface of water."<sup>14</sup>

That the solution of isoelectric casein in NaOH has no connection with the Donnan equilibrium is also evidenced by the fact that there is no point in the concentration of NaOH where further increase in the concentration of NaOH lowers the rate of solution, as would be the case if the Donnan equilibrium influenced this phenomenon.

The stability of solutions of this kind is guaranteed by the forces of chemical attraction between certain groups of the molecule of Na caseinate and water; and the precipitation by electrolytes is due to a diminution of these forces.

#### *IV. Precipitation and Solution of Gelatin in Water.*

Solutions of gelatin in water require enormous concentrations of salts for precipitation and it is very probable that the forces causing solution are the forces of residual valency discussed in the case of the solution of Na caseinate. These cases have no connection with the Donnan equilibrium, and this is borne out by the well known fact that solutions of gelatin are always more readily salted out by sulfates than by chlorides, regardless of the pH of the gelatin solution. This is illustrated by Table III.

0.8 per cent solutions of gelatin were prepared at three different pH; namely, 4.7 (isoelectric gelatin), 3.8 (gelatin chloride), and 6.4 to 7.0 (Na gelatinate). The purpose was to find the molecular concentration of different salts, namely,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{MgSO}_4$ ,  $\text{KCl}$ , and  $\text{MgCl}_2$ , required for precipitation. Table III shows that regardless of the pH the sulfates are better precipitants than the chlorides. There is, therefore, no definite relation between the sign of charge of the colloidal particles and of the precipitating ion.

<sup>14</sup> Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1850.

Precipitation experiments give no insight into the reason why sulfates are superior to chlorides in salting out; and an attempt was made to get an answer by experiments on the solution of gelatin. Such experiments gave the result that salts increase the solubility of isoelectric gelatin, and the more, the higher their concentration until finally a concentration is reached where the reverse effect is noticed. This reversal occurs at a comparatively low concentration in the case of the sulfates, while in the case of salts like  $\text{CaCl}_2$  or  $\text{MgCl}_2$  it does not seem to occur at all in the limit of the concentrations tried. The experiments were carried on at a temperature of  $35^\circ\text{C}$ .

Powdered gelatin of not too small a size of grain (going through sieve 30 but not through sieve 60) was rendered isoelectric in the way described in previous articles, and 0.8 gm. was put into 100 cc. of each of a series of solutions of  $\text{NaCl}$ ,  $\text{CaCl}_2$ , or  $\text{Na}_2\text{SO}_4$  varying in con-

TABLE III.

*Minimal Molar Concentrations Required to Precipitate 0.8 Per Cent Solutions of Gelatin.*

pH of gelatin solution.	Approximate molecular concentration of salt required for precipitation.				
	$(\text{NH}_4)_2\text{SO}_4$	$\text{Na}_2\text{SO}_4$	$\text{MgSO}_4$	$\text{KCl}$	$\text{MgCl}_2$
4.7.....	15/16 M	6/8 M	10/8 M	>3 M	>3 M
3.8 (gelatin chloride).....	13/16 M	5/8 M	7/8 M	3 M	>3 M
6.4 to 7.0 (Na gelatinate).....	16/16 M	7/8 M	9/8 M	>3 M	>3 M

centration from  $\text{M}/4096$  to 2 M. The suspensions of the powdered gelatin were frequently stirred and the time required to practically completely dissolve all the grains of powdered gelatin in suspension at  $35^\circ\text{C}$ . was measured. We consider this time the reciprocal of the solubility. The ordinates in Fig. 4 are the solution times (*i.e.*, the reciprocal of the solubility) of isoelectric gelatin, and the abscissæ are the molecular concentrations of the salt used. It is obvious that  $\text{NaCl}$  and still more  $\text{CaCl}_2$  increase the solubility (or diminish the solution time) of isoelectric gelatin in water, and the more, the higher the concentration of the salts. There exists a striking discontinuity in the  $\text{Na}_2\text{SO}_4$  curve. As long as the concentration of  $\text{Na}_2\text{SO}_4$  is below  $\text{M}/32$  it increases the solubility of gelatin, and the more so, the higher the concentration. When, however, the concentration

of  $\text{Na}_2\text{SO}_4$  is above  $M/32$ , a further increase in the concentration of  $\text{Na}_2\text{SO}_4$  diminishes the solubility of gelatin, and the more so, the higher the concentration of  $\text{Na}_2\text{SO}_4$ .  $(\text{NH}_4)_2\text{SO}_4$  acts in the same way. We now understand why it is that we cannot precipitate solutions of isoelectric gelatin with  $\text{KCl}$  or  $\text{MgCl}_2$  in concentrations

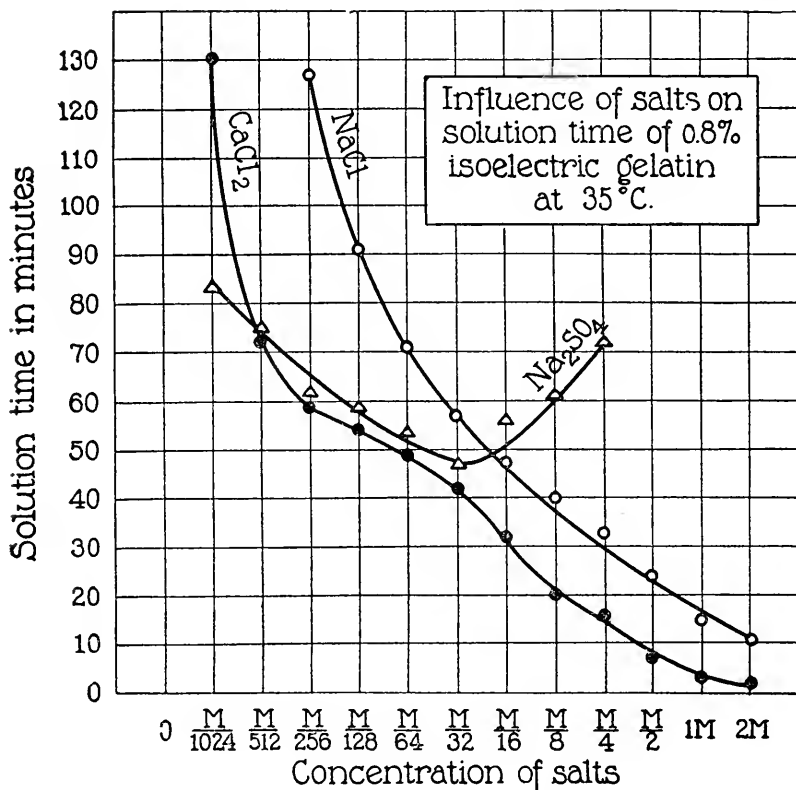


FIG. 4. Influence of salts on the time required to completely dissolve 0.8 gm. of powdered isoelectric gelatin in 100 cc. of salt solution of different concentrations at 35°C. Abscissæ are the concentrations of the salt; ordinates, the time required for complete solution.

up to 3 M (see Table III) while we can precipitate such solutions with sulfates but only at concentrations above  $M/2$ ; since our curve shows that at such high concentrations of sulfates the solubility of isoelectric gelatin in  $\text{Na}_2\text{SO}_4$  becomes less than in pure water.

While isoelectric gelatin is only sparingly soluble, gelatin salts

are highly soluble. 0.8 gm. of powdered gelatin of pH of about 3.3 dissolves very rapidly in 100 cc. HCl of the same pH at 35°C. The addition of NaCl or  $\text{CaCl}_2$  no longer increases the solubility, except when the concentration of  $\text{CaCl}_2$  becomes greater than  $M/16$ .  $\text{Na}_2\text{SO}_4$

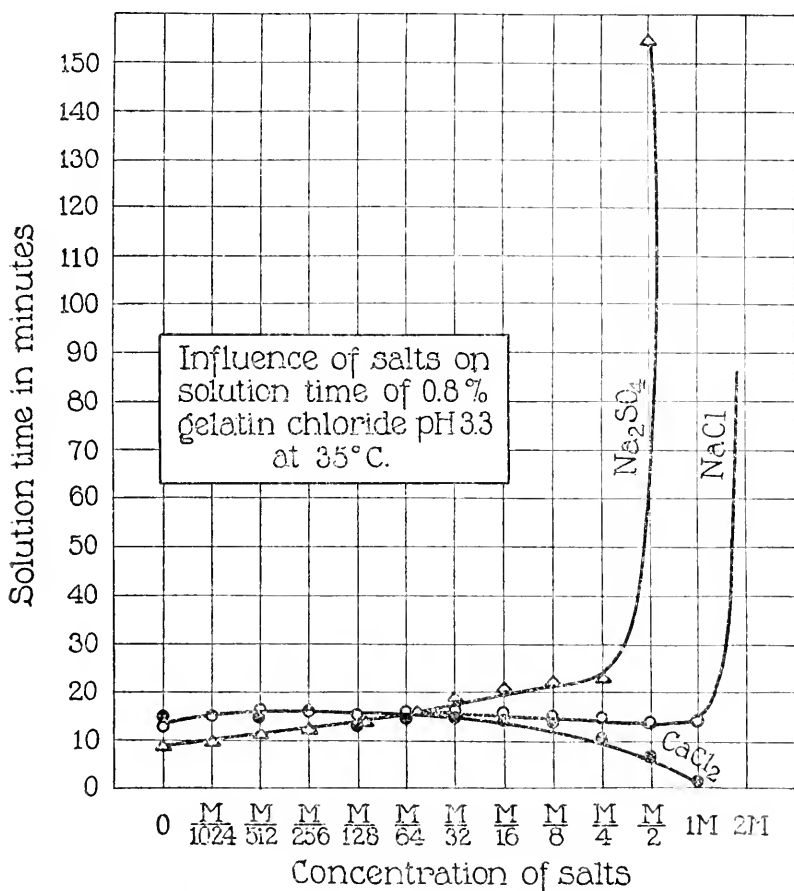


FIG. 5. Influence of salts on time required to dissolve 0.8 gm. of powdered gelatin chloride of pH 3.3 at 35°C.

abruptly diminishes the solubility at a concentration above  $M/4$  and NaCl above a concentration of 1 M (Fig. 5).

Fig. 6 shows the influence of the three salts on the solution time of Na gelatinate of pH 10.5.  $\text{Na}_2\text{SO}_4$  diminishes the solubility abruptly

at a concentration above  $M/8$ , while both  $\text{NaCl}$  and  $\text{CaCl}_2$  increase the solubility of  $\text{Na}$  gelatinate,  $\text{NaCl}$  in concentrations above  $M/2$ , and  $\text{CaCl}_2$  in concentrations above  $M/16$ .

In all three cases, therefore, is the solubility of gelatin diminished by sulfates, but only exceptionally by chlorides. This explains the

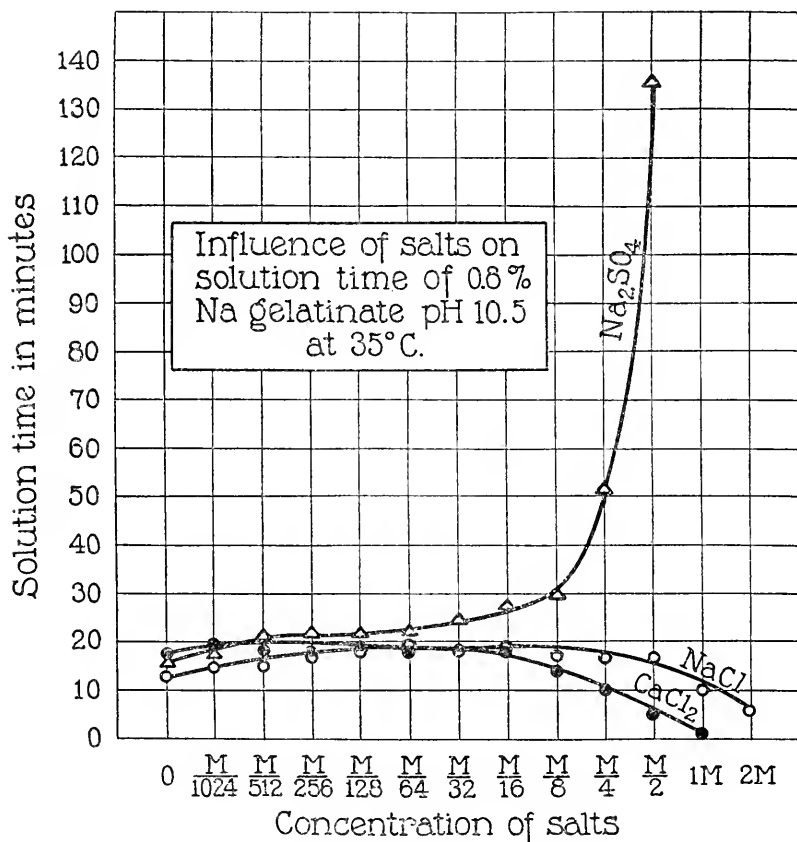


FIG. 6. Influence of salts on the time required to dissolve 0.8 gm. of powdered  $\text{Na}$  gelatinate of pH 10.5 at 35°C.

results contained in Table III.

We have seen that addition of little acid to isoelectric gelatin increases the osmotic pressure, viscosity, p.d., and swelling with increasing concentration, while beyond a certain pH the addition of more acid has a depressing effect. This is characteristic of the

Donnan effect. It was of interest to find out whether such a maximum followed by a drop existed in the influence of acid on the solubility of gelatin, but this is not the case at least between pH 4.7 and 1.0. Measurements of the dry weight of gelatin dissolved in a certain time at different pH, showed that the amount of gelatin dissolved increases with the hydrogen ion concentration. This corroborates the conclusion that the solution (and precipitation) of gelatin in water is not influenced by forces governed by the Donnan equilibrium and does therefore not show the characteristics of colloidal behavior.

These experiments also contradict the suggestion that the solution of solid gelatin is necessarily preceded by swelling and that swelling and solution are continuous processes. The contradiction lies in the fact that swelling in acid reaches a maximum at pH of about 3.0 and then diminishes upon further increase in hydrogen ion concentration, while the rate of solution of solid gelatin granules continues to increase steadily when the hydrogen ion concentration increases beyond pH of 3.0. The mechanism of swelling and the mechanism of solution of solid gelatin in water are determined by forces of an entirely different character; the swelling by osmotic pressure, and the solution by the secondary valency forces responsible for the solution of crystalloids.

#### *V. Solubility and Viscosity of Gelatin Solutions.*

We assumed in a preceding paper that the increase in the viscosity of gelatin solutions on standing is due to the gradual formation of larger aggregates from originally isolated gelatin molecules or gelatin ions. When we melt a solid gel of gelatin by rapidly heating it to 45°C. and cooling it rapidly to 20° there is produced a true solution containing isolated gelatin molecules or gelatin ions side by side with submicroscopic pieces of solid jelly. On standing two opposite processes are constantly going on in such a mixture; *i.e.*, the solution of these aggregates into isolated molecules or ions and the reverse process; namely, that of the formation of new aggregates by the union of formerly isolated gelatin molecules or gelatin ions. When the velocity of aggregate formation prevails over the velocity of the solution of aggregates, the viscosity of the gelatin solution will increase on standing; when the velocity of solution of aggregates

prevails over the velocity of formation, the viscosity of the gelatin solution will diminish on standing. All agencies which accelerate the rate of solution of solid gelatin should counteract the aggregate formation and the rise of viscosity of a gelatin solution on standing; while all agencies which diminish the rate of solution of solid gelatin should increase the rise of viscosity of the gelatin solution on standing.

We have seen that powdered isoelectric gelatin is dissolved the more rapidly, the more HCl we add, and that there is no maximum followed by a drop when a certain hydrogen ion concentration is exceeded. Hence we should expect that the lower the pH of a gelatin solution containing the same concentration of originally isoelectric gelatin, the smaller the increase of the viscosity of the solution on standing. In a preceding paper we have already given curves showing that this is the case and that there is no Donnan effect noticeable in this case.<sup>15</sup>

We have seen in Fig. 5 that  $\text{Na}_2\text{SO}_4$  commences to diminish noticeably the rate of solution of solid gelatin chloride as soon as the concentration of  $\text{Na}_2\text{SO}_4$  exceeds  $M/64$ , while  $\text{CaCl}_2$  commences to have the opposite effect as soon as the concentration of  $\text{CaCl}_2$  exceeds  $M/4$ .

We prepared gelatin chloride solutions of pH 3.4 containing 1 gm. of originally isoelectric gelatin in 100 cc. solution. The solutions were made up in various concentrations of  $\text{Na}_2\text{SO}_4$  and  $\text{CaCl}_2$ . The solutions were rapidly heated to  $45^\circ$ , rapidly cooled to  $20^\circ\text{C}.$ , and kept at this temperature for 1 hour. The time of outflow of the solution through a viscometer was measured immediately and in intervals of 5 or 10 minutes. The time of outflow of water through the viscometer at  $20^\circ$  was 61 seconds.

The viscosity of a gelatin chloride solution of pH 3.4 rises gradually but very slowly (uppermost curve in Fig. 7) and the rate of increase of viscosity on standing is not materially altered in  $M/512$   $\text{Na}_2\text{SO}_4$  and only little in  $M/128$   $\text{Na}_2\text{SO}_4$ . In  $M/32$   $\text{Na}_2\text{SO}_4$  the viscosity increases more rapidly on standing, in  $M/8$   $\text{Na}_2\text{SO}_4$  still more rapidly, and in  $M/2$   $\text{Na}_2\text{SO}_4$  very sharply. This is exactly what we should expect since the  $\text{Na}_2\text{SO}_4$  causes a diminution of the rate of solution of gelatin chloride as soon as the concentration of

<sup>15</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 97.



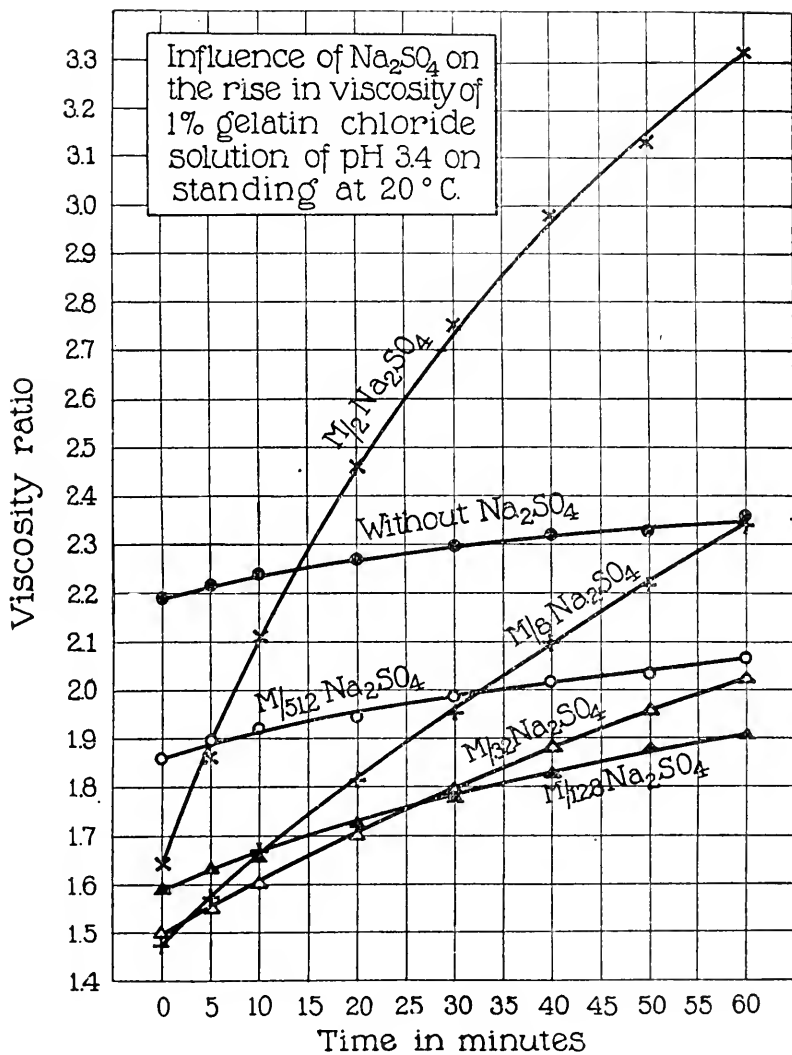


FIG. 7. Showing that concentrations of  $\text{Na}_2\text{SO}_4$  of  $M/32$  and above cause an increase in the viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

$\text{Na}_2\text{SO}_4$  is above  $M/64$ . In such solutions the rate of solution of micella will be less and less, and since new micella are constantly formed at  $20^\circ\text{C}$ ., the viscosity will rise more rapidly on standing when the solution contains  $\text{Na}_2\text{SO}_4$  in concentrations above  $M/64$  than when the solution contains no or less  $\text{Na}_2\text{SO}_4$ .

Fig. 8 shows that  $\text{CaCl}_2$  in concentrations up to  $M/8$  does not alter

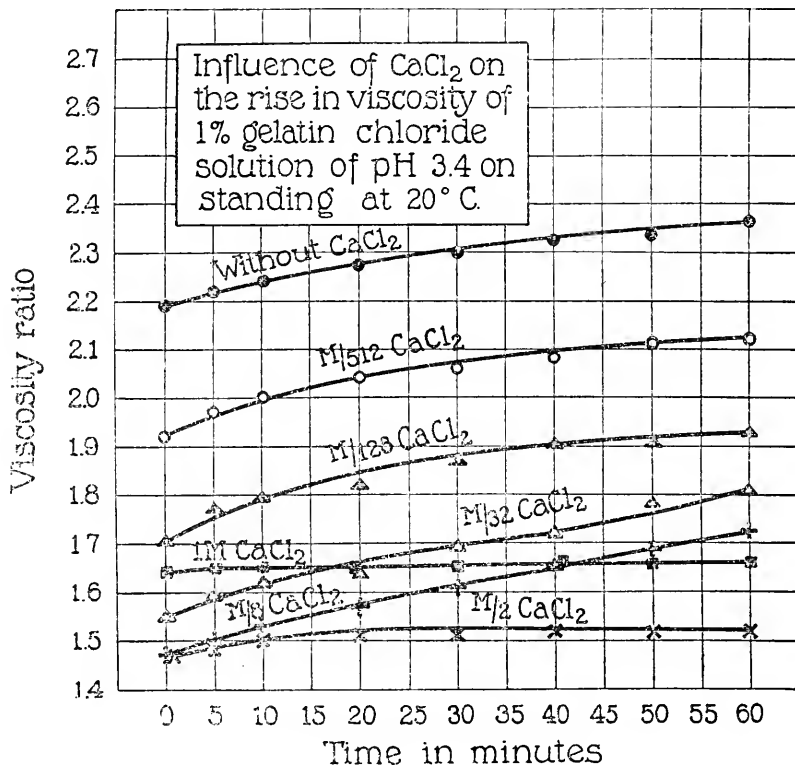


FIG. 8. Showing that concentrations of  $\text{CaCl}_2$  of  $M/2$  or above prevent the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at  $20^\circ\text{C}$ .

the increase in viscosity of gelatin chloride solution on standing but that the viscosity of gelatin chloride of pH 3.4 does no longer increase on standing when the concentration of  $\text{CaCl}_2$  is  $M/2$  or 1  $M$ . In this concentration  $\text{CaCl}_2$  causes a slight increase in the rate of solution of gelatin chloride.

NaCl causes no change in the rate of solution of gelatin chloride as long as the concentration of NaCl does not exceed 1 M. Above this concentration it causes coagulation and the viscosity can no longer be measured. Hence NaCl in concentrations up to 1 M should

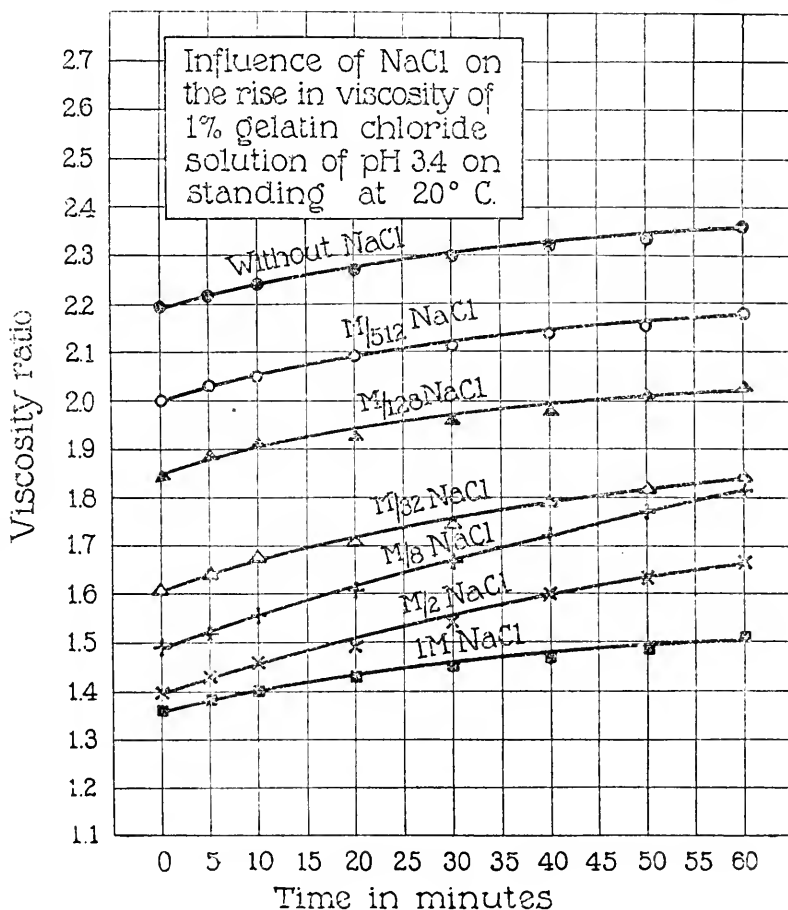


FIG. 9. Showing that NaCl solutions up to a concentration of 1 M have no effect on the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

not alter the rate of increase of viscosity of gelatin chloride solutions on standing. Fig. 9 shows that this is correct.

These experiments corroborate the idea that gelatin solutions

are true solutions consisting of isolated gelatin ions and molecules which may contain, in addition, submicroscopic aggregates of gelatin molecules and ions. The formation of new micella and a solution of these already formed occur constantly. When the rate of formation of such micella exceeds the rate of the solution, the viscosity of a gelatin solution rises on standing and the rise is in proportion with the difference in the rate of the formation and solution of these micella. The reader must bear in mind that the micella in the case of gelatin solutions are submicroscopic particles of a reversible jelly of gelatin and not particles of an irreversible precipitate.

#### SUMMARY.

1. Colloids have been divided into two groups according to the ease with which their solutions or suspensions are precipitated by electrolytes. One group (hydrophilic colloids), *e.g.*, solutions of gelatin or crystalline egg albumin in water, requires high concentrations of electrolytes for this purpose, while the other group (hydrophobic colloids) requires low concentrations. In the latter group the precipitating ion of the salt has the opposite sign of charge as the colloidal particle (Hardy's rule), while no such relation exists in the precipitation of colloids of the first group.

2. The influence of electrolytes on the solubility of solid Na caseinate, which belongs to the first group (hydrophilic colloids), and of solid casein chloride which belongs to the second group (hydrophobic colloids), was investigated and it was found that the forces determining the solution are entirely different in the two cases. The forces which cause the hydrophobic casein chloride to go into solution are forces regulated by the Donnan equilibrium; namely, the swelling of particles. As soon as the swelling of a solid particle of casein chloride exceeds a certain limit it is dissolved. The forces which cause the hydrophilic Na caseinate to go into solution are of a different character and may be those of residual valency. Swelling plays no rôle in this case, and the solubility of Na caseinate is not regulated by the Donnan equilibrium.

3. The stability of solutions of casein chloride (requiring low concentrations of electrolytes for precipitation) is due, first, to the osmotic pressure generated through the Donnan equilibrium between

the casein ions tending to form an aggregate, whereby the protein ions of the nascent micellum are forced apart again; and second, to the potential difference between the surface of a micellum and the surrounding solution (also regulated by the Donnan equilibrium) which prevents the further coalescence of micella already formed. This latter consequence of the Donnan effect had already been suggested by J. A. Wilson.

4. The precipitation of this group of hydrophobic colloids by salts is due to the diminution or annihilation of the osmotic pressure and the P.D. just discussed. Since low concentrations of electrolytes suffice for the depression of the swelling and P.D. of the micella, it is clear why low concentrations of electrolytes suffice for the precipitation of hydrophobic colloids, such as casein chloride.

5. This also explains why only that ion of the precipitating salt is active in the precipitation of hydrophobic colloids which has the opposite sign of charge as the colloidal ion, since this is always the case in the Donnan effect. Hardy's rule is, therefore, at least in the precipitation of casein chloride, only a consequence of the Donnan effect.

6. For the salting out of hydrophilic colloids, like gelatin, from watery solution, sulfates are more efficient than chlorides regardless of the pH of the gelatin solution. Solution experiments lead to the result that while  $\text{CaCl}_2$  or  $\text{NaCl}$  increase the solubility of isoelectric gelatin in water, and the more, the higher the concentration of the salt,  $\text{Na}_2\text{SO}_4$  increases the solubility of isoelectric gelatin in low concentrations, but when the concentration of  $\text{Na}_2\text{SO}_4$  exceeds  $M/32$  it diminishes the solubility of isoelectric gelatin the more, the higher the concentration. The reason for this difference in the action of the two salts is not yet clear.

7. There is neither any necessity nor any room for the assumption that the precipitation of proteins is due to the adsorption of the ions of the precipitating salt by the colloid.



# THE ORIGIN OF THE POTENTIAL DIFFERENCES RESPONSIBLE FOR ANOMALOUS OSMOSIS.

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## *I. Potential Differences in Collodion Membranes Coated with Protein.*

In a series of papers<sup>1</sup> the writer has published the results of experiments showing the peculiar influence of electrolytes on the rate of diffusion of water through collodion membranes. When a collodion membrane separates a solution of an electrolyte (of not too high a concentration) from pure water, the water diffuses from the side of pure water to the solution side, but the initial rate at which this diffusion occurs does not obey van't Hoff's law. The initial rate of diffusion was measured by the rise of level of liquid in a glass tube inserted through a rubber stopper in a closed collodion bag containing the solution, the collodion bag dipping into a beaker filled with pure water. When the membrane was merely a collodion membrane, the following rules expressed the influence of electrolytes on the initial rate of diffusion of water through the membrane from the water side to the side of the solution.

1. Water diffuses into the solution of an electrolyte through a collodion membrane as if the particles of water were positively charged and as if they were attracted by the anion and repelled by the cation of the electrolyte in solution with a force increasing with the valency.

2. In the case of certain electrolytes the "attracting force" of the anion for water increases at first more rapidly with increasing concentration than the "repelling force" of the cation, until a point is reached where with a further increase in concentration the "repelling force" of the cation increases more rapidly than the "attractive force" of the anion. Finally, a concentration is reached where the

<sup>1</sup>Loeb, J., *Science*, 1921, liii, 77; *J. Gen. Physiol.*, 1918-19, i, 717; 1919-20, ii, 173, 387, 577.

“attracting” and “repelling” forces balance each other and from then on the “attractive” force of the solution increases with the concentration, apparently in accordance with van’t Hoff’s law.

When, however, the collodion membrane has received a coating of a protein, *e.g.*, gelatin, the hydrogen ion concentration influences the

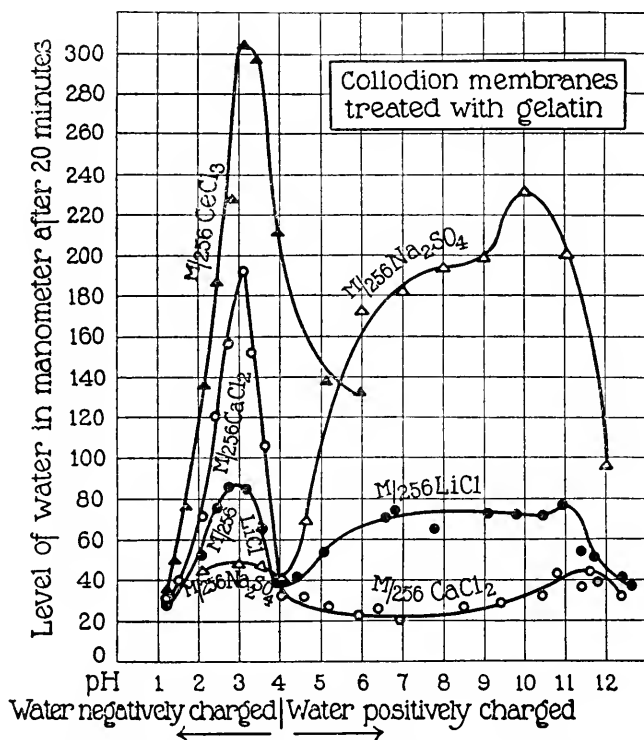


FIG. 1. Influence of hydrogen ion concentration on the initial rate of diffusion of liquid from a solution of  $\text{HNO}_3$  or  $\text{NaOH}$  respectively through a gelatin-coated membrane of collodion into a  $\text{M}/256$  solution of  $\text{LiCl}$ ,  $\text{CaCl}_2$ ,  $\text{CeCl}_3$ , or  $\text{Na}_2\text{SO}_4$  of the same hydrogen ion concentration. Abscissæ are the initial pH; ordinates, the rise of level of liquid in manometer connected with the salt solution, after 20 minutes.

initial rate of diffusion of water from pure water into the solution in a still more complicated way (Fig. 1). The procedure in these experiments was as follows: Both the outside solution as well as the inside solution had the same hydrogen ion concentration,



containing the same concentration of  $\text{HNO}_3$  or  $\text{NaOH}$  respectively. In addition the inside solution (*i.e.*, the solution inside the collodion bag which was connected with the manometer) contained a M/256 solution of one of the following four salts:  $\text{CeCl}_3$ ,  $\text{CaCl}_2$ ,  $\text{LiCl}$ , and  $\text{Na}_2\text{SO}_4$ . The "attraction" of these M/256 salt solutions for water was therefore tested at different hydrogen ion concentrations. It is obvious from Fig. 1 that the "attractive" force of one and the same salt solution for water (measured by the initial rate of diffusion of water from the outside into the salt solution) varied considerably with the hydrogen ion concentration of the solution. (The hydrogen ion concentration is expressed by Sørensen's logarithmic symbol pH; *i.e.*, the log of the concentration with the minus sign omitted). The abscissæ in the curve are the initial pH of the solutions, while the ordinates are the rise in level of the watery liquid in the glass tubes after 20 minutes at  $24^\circ\text{C}$ .

Fig. 1 shows that somewhere between pH 4.0 and 5.0 a reversal of the sign of charge of the water particles occurs. At pH below 4.0 the water is negatively charged, at pH above 5.0, it is positively charged. This change coincides with a change in the nature of the charge of the gelatin ion. At a pH of 4.0 or below the gelatin forms gelatin nitrate (in the presence of  $\text{HNO}_3$ ) and hence the gelatin ion is a cation. The water, being negative, has the opposite sign of charge as the gelatin ion. At a pH of 5.0 or above the gelatin forms metal gelatinate, Na gelatinate, Ca gelatinate, etc., and the gelatin ion is negatively charged. The water, being positively charged, has again the opposite charge as the gelatin ion. The exact turning point, the isoelectric point of gelatin, is at a pH 4.7. In a preceding paper<sup>2</sup> the writer has been able to show that if other proteins than gelatin are used to give the collodion membrane a coating of a protein, the reversal of the sign of charge of the water varies with the isoelectric point of the protein used.

If we return to Fig. 1 we notice that where the water is negatively charged it is "attracted" by the cation of the salt used and "repelled" by the anion with a force increasing with the valency of the ion. The attractive force increases from Li to Ca to Ce, and diminishes from

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 577.

Cl to  $\text{SO}_4$ . Where the water is positively charged the reverse order of efficiency exists.

The fact that the same concentration of the salts, namely,  $\text{M}/256$ , was used, has no essential influence on the results. Similar results were obtained when the salt solutions had the osmotic pressure of a  $\text{M}/64$  solution of cane sugar.

Those who have studied phenomena of abnormal osmosis through membranes, Girard,<sup>3</sup> Bernstein,<sup>4</sup> Bartell and his collaborators,<sup>5</sup> and Freundlich<sup>6</sup> have reached the conclusion that potential differences on the opposite sides of the membrane are responsible for these anomalies in diffusion. The fact that the sign of charge varies in these experiments with the sign of charge of the protein ion gives a favorable point of attack for the investigation of the origin of the potential differences. In order to simplify the experiments they were confined to pH from 4.6 to pH 1.9; *i.e.*, to that region where the water diffuses as if it were negatively electrified. The method of procedure was as follows.  $\text{M}/256$  solutions of one of the four salts mentioned were prepared in different concentrations of  $\text{HNO}_3$  and put into collodion bags of about 50 cc. volume, which were lined by a film of gelatin on the inside. These bags were closed with a perforated rubber stopper through which was pushed a glass tube with a diameter of about 2 mm. to indicate the rise of liquid. The collodion bags were put into beakers containing 350 cc. of the same concentration of  $\text{HNO}_3$  as that inside the bag. Fig. 2 gives the rise of the level of liquid in the manometer in one set of experiments after 1 hour at  $24^\circ\text{C}$ . The abscissæ are the initial pH of the liquid which was the same inside and outside, the ordinates are the rise of the level of liquid after 1 hour. It is obvious in this case that the curves have a minimum near the isoelectric point of gelatin, that the rate of diffusion of water into the solution rises

<sup>3</sup> Girard, P., *Compt. rend. Acad.*, 1908, cxlvi, 927; 1909, cxlviii, 1047, 1186; 1910, cl, 1446; 1911, clii, 401; La pression osmotique et le mécanisme de l'osmose, Publications de la Société de Chimie-physique, Paris, 1912.

<sup>4</sup> Bernstein, J., *Elektrobiologie*, Braunschweig, 1912.

<sup>5</sup> Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036. Bartell, F. E., and Madison, O. E., *J. Physical Chem.*, 1920, xxiv, 593.

<sup>6</sup> Freundlich, H., *Kolloid. Z.*, 1916, xviii, 11.

with the increasing hydrogen ion concentration reaching a maximum at pH 3.6 or 3.0 respectively, and that the rate of diffusion falls again with a further increase in the hydrogen ion concentration.  $M/256$   $\text{Na}_2\text{SO}_4$  shows little "attraction" for water, and  $M/128$  cane sugar practically none.

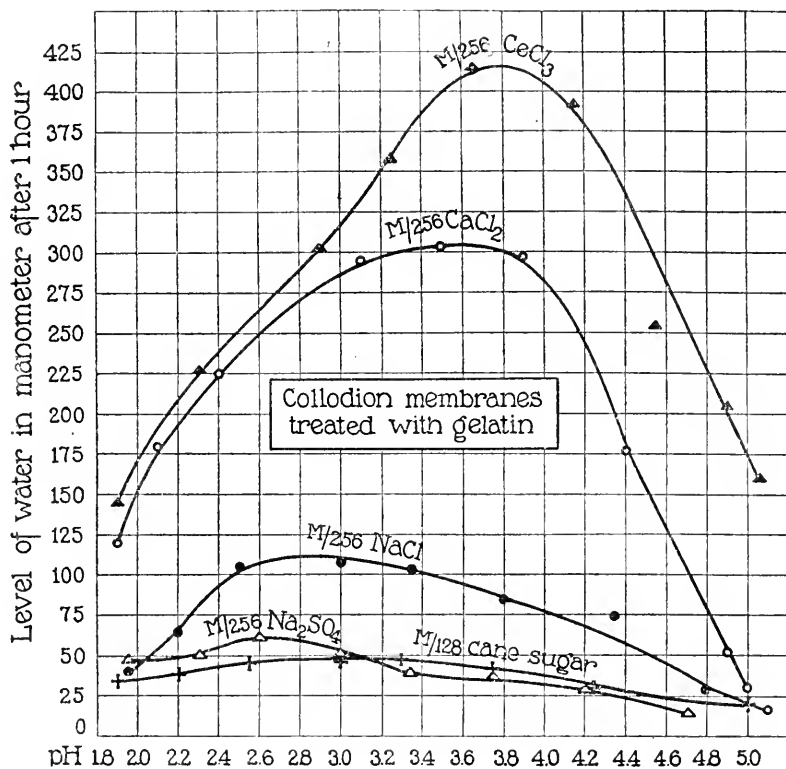


FIG. 2. Influence of hydrogen ion concentration on initial rate of diffusion of liquid from a solution of  $\text{HNO}_3$  through a gelatin-coated collodion membrane into a  $M/128$  solution of cane sugar,  $M/256$   $\text{NaCl}$ ,  $\text{CaCl}_2$ ,  $\text{CeCl}_3$ , or  $\text{Na}_2\text{SO}_4$  of the same hydrogen ion concentration. Abscissæ are the pH; ordinates, rise of level of liquid in manometer connected with the salt solution, after 1 hour. Water negatively charged.

The writer has recently measured the potential differences between the inside salt solution and the outside solution at the end of an hour with the aid of a Compton electrometer (calomel electrodes with

saturated KCl solution) and obtained a set of curves presented in Fig. 3. At this time some of the salt had diffused from the inside into the outside solution. The p.d. curves in Fig. 3 resemble the osmotic curves in Fig. 2 sufficiently to suspect a connection

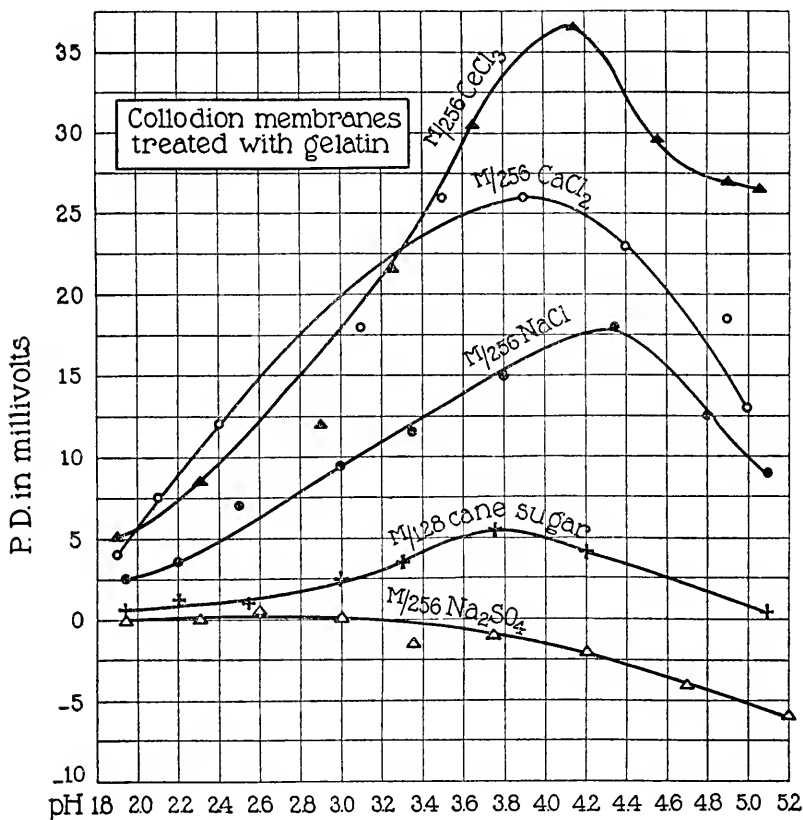


FIG. 3. Potential difference between salt solutions and external solution in preceding experiment after 1 hour of diffusion. Abscissæ are the pH at beginning of experiment; ordinates, p.d. at the end of the experiment. Attention is called to a certain similarity of the curves in Figs. 2 and 3.

between the two, so that we may assume that the p.d. between the opposite sides of the membrane is the main driving force which (for low concentrations of electrolytes) moves the water from the water side to the solution side of the membrane.

The next question was: What is the origin of the P.D.? It occurred to the writer that a measurement of the hydrogen ion concentration inside and outside after 1 hour might possibly throw a light on this problem. The results of these measurements are plotted in Fig. 4. The reader will remember that at the beginning the hydrogen ion concentration was the same inside and outside. Yet after 1 hour

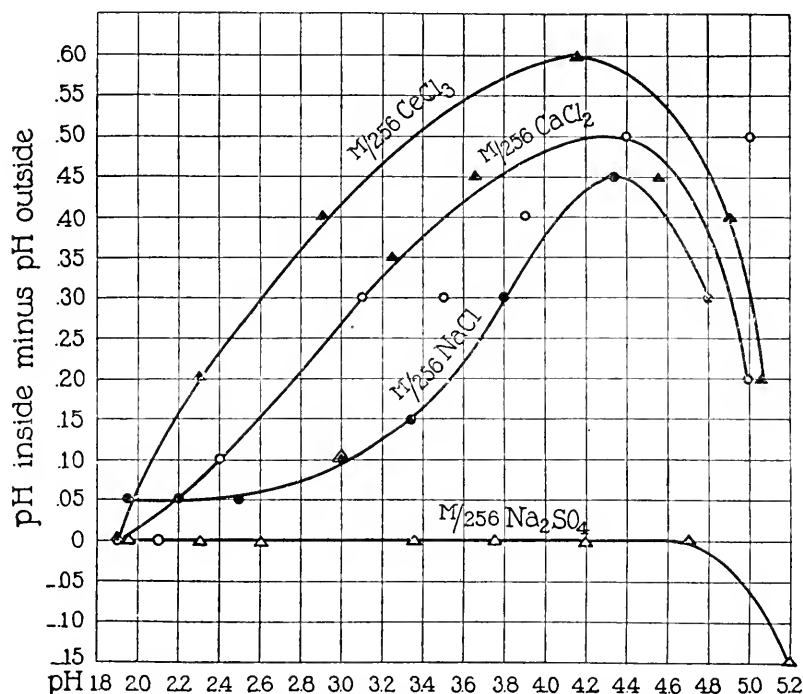


FIG. 4. Abscissæ are the initial pH; ordinates, the values (pH inside minus pH outside) in preceding experiment. Attention is called to the similarity of the curves in Figs. 3 and 4.

(and in fact much earlier) it is obvious that some of the acid has diffused from the inside into the outside solution so that the hydrogen ion concentration is higher outside than inside the collodion bag containing the salt solution.

This then leads to the result that when we separate identical acid solutions by a collodion membrane coated with a film of gelatin, and put inside the collodion bag a solution of a neutral salt with a mono-

valent anion (e.g., Cl), acid is driven from the side where the salt is added to the side originally free from salt. This difference in the pH inside minus pH outside gives rise to a potential difference which was discussed in a preceding paper.<sup>7</sup> In this paper it was shown that when we separate a gelatin chloride solution from a solution of HCl (without gelatin) both having at the beginning the same hydrogen ion concentration, acid is forced from the gelatin solution into the outside solution. The same happens when solid jelly of gelatin chloride is separated from a HCl solution. In this case there arises a P.D. between the two phases; and the writer has been able to show that this P.D. can be calculated with a good degree of accuracy from the difference of the hydrogen ion concentration inside and outside, on the basis of Nernst's well known formula. By multiplying the value (pH inside minus pH outside) by 58 we get the value for the P.D. actually observed at 18°C. in terms of millivolts.<sup>7</sup>

This unequal distribution of acid inside and outside is due to a peculiar membrane equilibrium the theory of which was developed by Donnan.<sup>8</sup> Our new experiments show that such an equilibrium condition is produced also between a gelatin membrane and a solution of HCl free from gelatin. The coating of gelatin on the collodion membrane behaves like a solid jelly of gelatin, the gelatin being transformed into gelatin chloride when in contact with HCl. The Donnan equilibrium demands that the concentration of free HCl inside the gelatin membrane be less than the concentration of free HCl of the solution bounding the membrane. This gives rise to the P.D. The value of pH inside minus pH outside is diminished when a neutral salt is added—in accordance with Donnan's theory—and the fact that a salt is added in our diffusion experiments on one side of the membrane but not on the other is the cause of the fact that the pH of the acid solution containing the salt solution (inside solution) becomes higher than the pH of the solution containing no or less salt (outside solution). The reader will find the experiments proving this in the writer's paper referred to.<sup>7</sup>

<sup>7</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 667.

<sup>8</sup> Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

A comparison of Figs. 3 and 4 shows that the two sets of curves, that for the P.D. (Fig. 3) and for the values  $\text{pH inside} - \text{pH outside}$  (Fig. 4) when plotted over the initial pH as abscissæ, have certain features in common. Both sets of curves have a minimum at or near the isoelectric point of gelatin ( $\text{pH } 4.7$ ). They both rise to a maximum at or not far from  $\text{pH } 4.2$ , and then drop again when the initial pH of the solution drops further. This parallelism suggests that this form of the P.D. curve is determined by the differences in the hydrogen ion concentration produced on the opposite sides of the membrane in conformity with Donnan's theory of membrane equilibrium.

## *II. Potential Differences in Collodion Membranes Free from Protein.*

The experiments just described were repeated with collodion membranes which had not been treated with a protein. The difference between the P.D. in this case (Fig. 5) and the P.D. observed in the case of collodion membranes coated with a film of gelatin (Fig. 3) is striking. The method of experimentation was the same in the case of the collodion bags free from gelatin as in the experiments of the preceding chapter. The initial concentration of  $\text{HNO}_3$  was always the same inside and outside. The inside solution, however, contained in addition to the acid a solution of  $\text{M}/256$  of one of the four salts,  $\text{CeCl}_3$ ,  $\text{CaCl}_2$ ,  $\text{NaCl}$ , and  $\text{Na}_2\text{SO}_4$ . After 1 hour the P.D. inside and outside was measured.

We notice that the P.D. is no longer a minimum at or near  $\text{pH}=4.0$  and we can therefore be certain that this feature of the curves in Fig. 3 was due to the gelatin. The curves in Figs. 3 and 5 have, however, one feature in common; namely, that the P.D. increases with the increasing valency of the cation and diminishes with the increasing valency of the anion. This feature is, therefore, not a specific function of the film of gelatin.

Since the water is positively charged even in the presence of acid when the membrane consists of collodion free from protein, it is to be expected that only the  $\text{Na}_2\text{SO}_4$  solution should attract the water, while no such attraction should occur in the case of  $\text{M}/256$   $\text{CeCl}_3$  or  $\text{M}/256$   $\text{CaCl}_2$ . Fig. 6 shows that this is true.

The question then arises: How can we account for this valency effect of the ion with the same sign of charge as that of the particle of water? The answer seems to be that we are dealing here with diffusion potentials which are independent of the membrane. This was proved

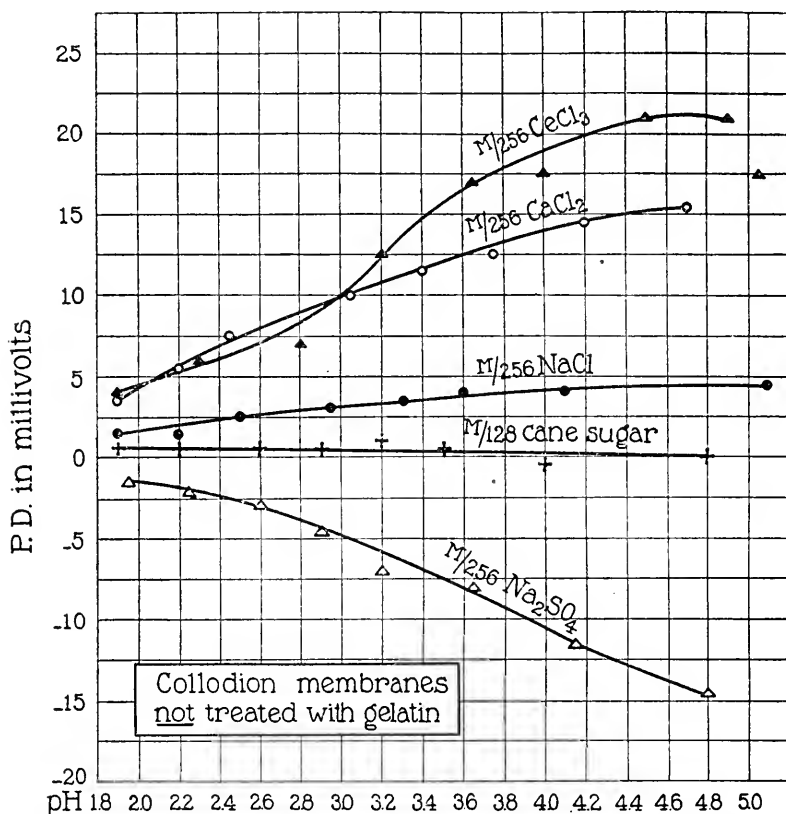


FIG. 5. Influence of neutral salts on the P.D. across collodion membranes not treated with gelatin. Otherwise same experiment as in Fig. 3. Notice difference between curves in Figs. 5 and 3, revealing the share of the protein in the origin of the P.D.

in the following way. Two beakers were filled with identical solutions of  $\text{HNO}_3$ , the  $\text{HNO}_3$  solution in one of the beakers being dissolved in  $\text{m}/256$   $\text{CeCl}_3$  or  $\text{CaCl}_2$  or  $\text{NaCl}$  or  $\text{Na}_2\text{SO}_4$ , while the  $\text{HNO}_3$  in the other was dissolved in pure water. The pH was identical in



the two solutions. They were connected by a bent glass tube, filled with a pure  $\text{HNO}_3$  solution, of the same pH as that in the two beakers. Into each beaker was dipped a calomel electrode with saturated  $\text{KCl}$  solution and the diffusion potential was ascertained with the aid of a Compton electrometer. Fig. 7 gives the P.D. A comparison between Figs. 5 and 7 shows that the curves representing the P.D. in the two cases are very similar.

If we now return to a discussion of the curves in Figs. 1 and 2, representing the influence of the pH on the attraction of a  $m/256$  solution of a neutral salt for water through a collodion membrane impregnated

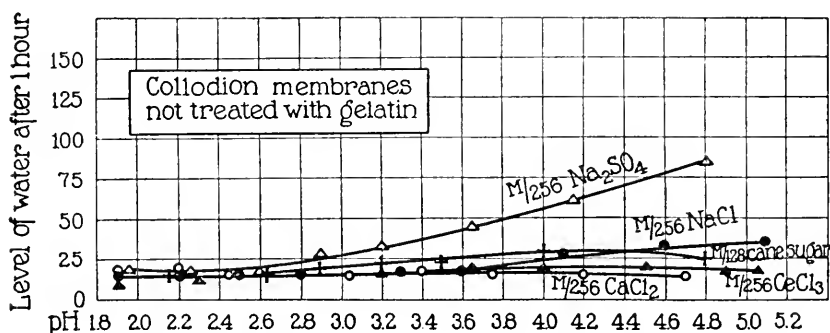


FIG. 6. Influence of  $m/128$  cane sugar,  $m/256 \text{ NaCl}$ ,  $\text{CaCl}_2$ ,  $\text{CeCl}_3$ , and  $\text{Na}_2\text{SO}_4$  on the initial rate of diffusion of water through collodion membranes not treated with gelatin. Otherwise the experiment is the same as in Fig. 2. Abscissæ are the pH; ordinates, rise of level of water in salt solution after 1 hour. Only  $\text{Na}_2\text{SO}_4$  attracts water to a noticeable amount since the latter is positively charged when diffusing through the membrane.

with gelatin, we can say that these curves resemble the curves in Fig. 3 for the P.D. on the opposite sides of the membrane.

Figs. 4 and 7 show that the P.D. curves in Fig. 3 have a double source. One is connected with the influence of the gelatin layer on the unequal distribution of the acid on the opposite sides of the membrane, which finds its explanation probably in the Donnan effect. The other source of the P.D. seems to be the diffusion potentials as shown in Fig. 7.

These diffusion potentials which exist regardless of the presence or absence of a membrane and regardless of the nature of the membrane seem to be responsible for the fact that the ions with opposite sign

of charge as that of the water "attract" the water with a force increasing with the valency of the ion, while the Donnan effect seems to be

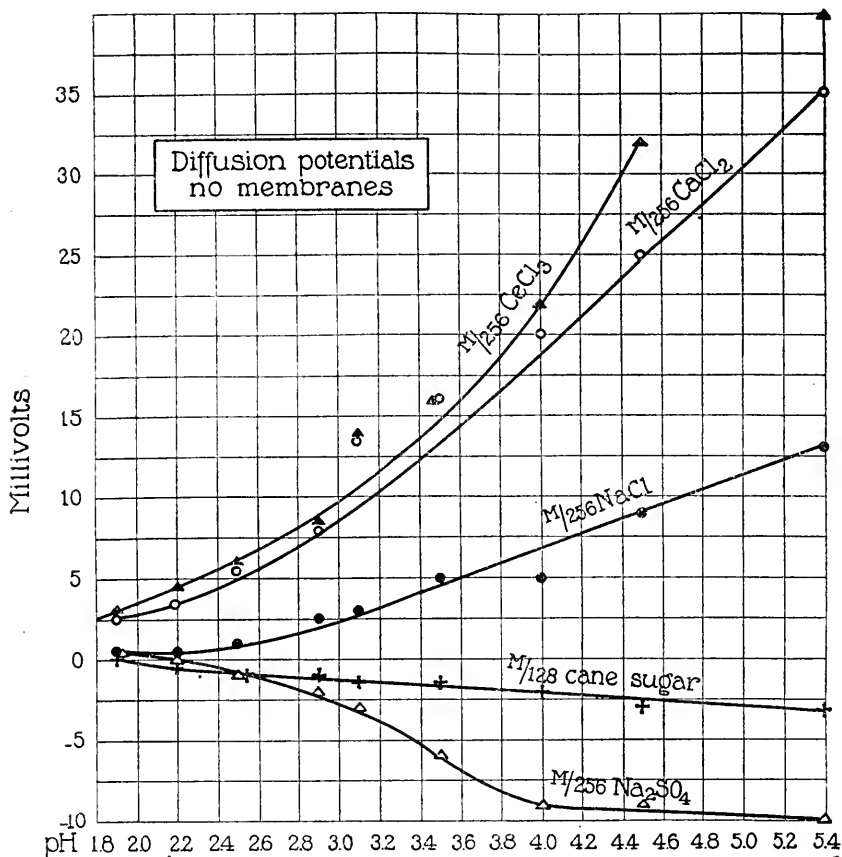


FIG. 7. Diffusion potentials between solutions of M/128 cane sugar, M/256 NaCl,  $\text{CaCl}_2$ ,  $\text{CeCl}_3$ , and  $\text{Na}_2\text{SO}_4$  in various concentrations of  $\text{HNO}_3$  and solutions free from these salts but of the same concentrations of  $\text{HNO}_3$ . The salt solutions and the solutions free from salt had always the same pH. Abscissæ are the pH; ordinates, diffusion potentials. Attention is called to the resemblance between the curves in Fig. 7 and those in Fig. 5.

responsible for the fact that when the collodion membrane is impregnated with a protein the rate of diffusion of the negatively charged water is a minimum at the isoelectric point of the protein, increasing

with a diminishing pH until a maximum is reached at a pH of about 4.2, and diminishing with a further diminution of the pH.

These experiments then lead to the result that the anomalous osmosis discussed in this paper is determined by a potential difference between the opposite sides of the membrane, and that this P.D. seems to have a double source; namely, the Donnan equilibrium and diffusion potentials.

In addition to these two sources of P.D. between the opposite sides of the membrane there may be others, as, *e.g.*, the Coehn effect due to the difference in the dielectric constant between liquid and membrane. If such a P.D. plays a rôle in our experiments it can be only of minor importance.

The experiments suggest the possibility that the establishment of a Donnan equilibrium between membrane and solution is one of the factors determining the Helmholtzian electrical double layer, at least in the conditions of our experiments.

#### SUMMARY AND CONCLUSION.

1. Collodion bags coated with gelatin on the inside were filled with a M/256 solution of neutral salt (*e.g.*, NaCl, CaCl<sub>2</sub>, CeCl<sub>3</sub>, or Na<sub>2</sub>SO<sub>4</sub>) made up in various concentrations of HNO<sub>3</sub> (varying from N/50,000 to N/100). Each collodion bag was put into an HNO<sub>3</sub> solution of the same concentration as that inside the bag but containing no salt. In this case water diffuses from the outside solution (containing no salt) into the inside solution (containing the salt) with a relative initial velocity which can be expressed by the following rules: (a) Water diffuses into the salt solution as if the particles of water were negatively charged and as if they were attracted by the cation and repelled by the anion of the salt with a force increasing with the valency of the ion. (b) The initial rate of the diffusion of water is a minimum at the hydrogen ion concentration of about N/50,000 HCl (pH 4.7, which is the point at which gelatin is not ionized), rises with increasing hydrogen ion concentration until it reaches a maximum and then diminishes again with a further rise in the initial hydrogen ion concentration.

2. The potential differences between the salt solution and the outside solution (originally free from salt) were measured after the

diffusion had been going on for 1 hour; and when these values were plotted as ordinates over the original pH as abscissæ, the curves obtained were found to be similar to the osmotic rate curves. This confirms the view expressed by Girard, Bernstein, Bartell, and Freundlich that these cases of anomalous osmosis are in reality cases of electrical endosmose where the driving force is a P.D. between the opposite sides of the membrane.

3. The question arose as to the origin of these P. D. and it was found that the P.D. has apparently a double origin. Certain features of the P.D. curve, such as the rise and fall with varying pH, seem to be the consequence of a Donnan equilibrium which leads to some of the free  $\text{HNO}_3$  being forced from the solution containing salt into the outside solution containing no (or less) salt. This difference of the concentration of  $\text{HNO}_3$ , on the opposite sides of the membrane leads to a P.D. which in conformity with Nernst's theory of concentration cells should be equal to  $58 \times (\text{pH inside minus pH outside})$  millivolts at  $18^\circ\text{C}$ . The curves of the values of  $(\text{pH inside minus pH outside})$  when plotted as ordinates over the original pH as abscissæ lead to curves resembling those for the P. D. in regard to location of minimum and maximum.

4. A second source of the P.D. seems to be diffusion potentials, which exist even if no membranes are present and which seem to be responsible for the fact that the rate of diffusion of negatively charged water into the salt solution increases with the valency of the cation and diminishes with the valency of the anion of the salt.

5. The experiments suggest the possibility that the establishment of a Donnan equilibrium between membrane and solution is one of the factors determining the Helmholtzian electrical double layer, at least in the conditions of our experiments.

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## THE INACTIVATION OF TRYPSIN. I.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 15, 1921.)

In most of the early work of the kinetics of enzyme action it was tacitly assumed that enzyme reactions obeyed the law of mass action and that they represented a special case of homogeneous catalysis. As the individual enzymes were studied more carefully, however, it became apparent that few, if any, obeyed the law of mass action in its simplest form. Several authors have, therefore, taken the view that enzyme reactions should be considered as occurring in heterogeneous systems, and that owing to their colloidal nature they do not conform to the law of mass action. On the other hand, Tammann, Michaelis, Arrhenius,<sup>1</sup> Euler,<sup>2</sup> Taylor,<sup>3</sup> and others consider that in general the law of mass action does hold and that the apparent divergencies are due to secondary reactions.<sup>4</sup> The author has found that the peculiarities of pepsin digestion may be accounted for in this way. It seems possible to explain the kinetics of an enzyme hydrolysis fairly well from either point of view. It is possible to find very close analogies on the one hand with ordinary homogeneous catalysis, and on the other hand with heterogeneous catalysis. From the theoretical side it has been stated that since most enzymes are admittedly colloidal it is not justifiable to apply the law of mass action to them. It may be pointed out, however, that the work of Perrin and of Svedberg<sup>4</sup> has shown that colloidal solutions obey the gas laws very well, in fact, much better than do ionized substances. Since the law of mass

<sup>1</sup> Arrhenius, S., *Quantitative laws in biological chemistry*, London, 1915.

<sup>2</sup> For a discussion of this question see von Euler, H., *Allgemeine Chemie der Enzyme*, 2nd edition, Munich, 1900.

<sup>3</sup> Taylor, A. E., On fermentation, *Univ. Calif. Pub., Pathology*, 1903-07, i, 244.

<sup>4</sup> Svedberg, T., *Z. physik. Chem.*, 1910, lxxiii, 547; Svedberg, T. and Inouye, K., 1911, lxxvii, 145. Lewis, W. C. McC., *A system of physical chemistry*, i, 1918.

action can be derived from the gas laws and the two laws of thermodynamics, there seems to be every reason to suppose that colloidal solutions should obey the law of mass action. The question appears to be one of experimental fact. If it is found that enzyme reactions may be accounted for on the basis of the law of mass action there seems to be no theoretical reason to disregard this fact and attempt an explanation from the point of view of adsorption. Most of the experimental evidence at hand, however, consists of data on the kinetics of the reactions. It is a matter of experience that conclusions based on kinetics alone are exceedingly uncertain especially when, as is the case with enzymes, the equations used contain at least two constants. It seems better therefore to attack the question from another angle. It is known that various substances retard the action of enzymes and that there must therefore be some kind of a reaction between these substances and the enzyme (or the substrate). If it could be shown that this reaction conformed accurately to the law of mass action it would furnish experimental justification for the application of this law to the enzyme reaction in general, at least as far as the particular enzyme is concerned. It has been shown by the author that the equilibrium between pepsin and the products formed by its action on proteins does conform quite accurately to the law of mass action.<sup>5</sup> Euler and Svanberg<sup>6</sup> have shown that the inactivation of invertase by various crystalloid substances is due to an equilibrium which is accurately expressed by the same law. The experiments described in this paper were undertaken with the view of determining whether or not the same condition is found in the case of trypsin. As will be seen from these experiments, the reaction between trypsin and the substances which inhibit its action may be accurately accounted for by the law of mass action.

It is known that various substances inhibit the action of trypsin. Bayliss<sup>7</sup> found that some, at least, of the products formed by the action of the enzyme on proteins inhibited its action and also rendered it more stable. He concluded, therefore, that they combined in some

<sup>5</sup> Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 471.

<sup>6</sup> von Euler, H., and Svanberg, O., *Fermentforschung*, 1919-20, iii, 330; 1921, iv, 142.

<sup>7</sup> Bayliss, W. M., *Arch. Biol.*, 1904, xi, Suppl. 261.

way with the enzyme. Bayliss considered that the amino-acids were probably responsible for this inhibiting effect. It would seem, therefore, that these substances are favorable for the purpose of the present experiments since they are also involved in the kinetics of the reaction, and are chemically well defined substances. It was found, however, that the amino-acids themselves have very little if any effect, if the effect on the pH was controlled, whereas the solution resulting from the hydrolysis of the protein by trypsin was strongly inhibitory. Such solutions were, therefore, used although it was not found possible to determine exactly what chemical compound was responsible for their reaction.

*Methods Used in the Present Investigation.*

Inasmuch as the quantity of an enzyme can only be determined by measuring the rate at which it reacts it is necessary to have some convenient and accurate method for following the course of the hydrolysis. In the present experiments it is also necessary to arrange conditions in such a way as to prevent any change in pH during the course of the hydrolysis. Henri and des Bancel<sup>8</sup> found that the hydrolysis of proteins by trypsin could be accurately followed by noting the change in the conductivity of the solution. The same method was used by Bayliss who also showed that the change in conductivity was not caused by changes in the viscosity of the solution and was parallel to the amount of non-precipitable nitrogen formed. A number of experiments were made to determine the best conditions to use for the purpose of the present experiments. It was found that at a pH of 6.2 to 6.4 no change occurred in the pH during the hydrolysis of gelatin (due probably to the fact that this is near the isoelectric point of the products formed), so that it was not necessary to use buffer solutions if the determinations were made in this range. This is a great advantage since buffers interfere with the determination of the conductivity. Gelatin was found to be the best protein since it gives clear solutions and is easily prepared in the necessary salt-free condition. Since trypsin is very unstable in solution the experiments were made at 33°C. The change in the conductivity of the solutions was followed by the change in the readings of the Kohlrausch bridge. These readings are proportional to the percentage change in the conductivity, so that the lower the original conductivity of the solution the greater will be the change in the bridge readings for a given absolute increase in the conductivity of the solution. On the other hand, the lower the original conductivity the more sensitive the solution is to the addition of traces of salts, to temperature change, etc. It was found better, therefore, to increase the conductivity of the gelatin by the addition of KCl until the solution had a specific conductivity at 33°C. of  $2 \times 10^{-3}$  reciprocal ohms.

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<sup>8</sup> Henri, V., and des Bancel, L., *Compt. rend. Biol.*, 1903, lv, 563, 787, 788.

*Experimental Procedure.*

*Preparation of the Gelatin.*—Salt-free gelatin was prepared as described by Loeb,<sup>9</sup> i.e., the gelatin was brought to the isoelectric point and then washed (except that 100 gm. were prepared at a time). The gelatin was melted, diluted to about 5 per cent and titrated to a pH of 6.3 with NaOH. It was then diluted so as to contain 2.5 gm. dry weight per 100 cc. and sufficient KCl added so that the resulting solution had a specific conductivity at 33°C. of  $2 \times 10^{-3}$  reciprocal ohms. A few crystals of thymol were added and the solution was kept in the ice box.

*Preparation of the Trypsin.*—The trypsin used in all the experiments was a sample of Fairchild's trypsin. It was prepared for use by suspending 5 gm. in 50 cc. of water and dialyzing under pressure at 6°C. for 18 hours. The solution was then filtered from the rather heavy precipitate and sufficient KCl added to bring the specific conductivity to  $2 \times 10^{-3}$ . This solution is very unstable and loses its activity quite rapidly even at 3°C. It was prepared fresh each day.

*Determination of the Conductivity.*—The apparatus used was a Leeds and Northrup Kohlrausch bridge and resistance box. The change in the bridge readings were used direct to avoid calculation. These readings are related to the actual conductivity by the formula

$$X = \frac{A}{1000 - A} R$$

in which  $X$  = resistance of solution,  $A$  = bridge reading, and  $R$  = resistance of standard resistance box. It will be seen that if the readings are always begun at the middle of the bridge (500), the first ten or fifteen points will each represent very nearly equal changes in the conductivity and may be considered as proportional to the percentage change in the conductivity. Since the conductivity increases the bridge readings will decrease. If all the solutions have the same conductivity at the beginning of the experiment and the resistance is so chosen that the bridge reading at the beginning is 500 in each case, then a change in the bridge reading of from 500 to 490 will represent the same change in the conductivity of each of the different solutions.

*Type of Conductivity Cell.*—The cell used is shown in Fig. 1. Fifteen such cells were made and adjusted (by warming the cement and moving the electrode) so that they all had the same constant, 3.5. The electrodes were plated with platinum black. Readings could be made with ease to half a scale division on the bridge. As little current as possible was run through the cell, although no effect due to the passage of current could be noted.

*pH Determinations.*—The determinations were made by the E. M. F. method.

*Formol Titration.*—The titration was carried out as described in a previous paper<sup>10</sup> by adjusting the solution to pH 7.0 with neutral red as an indicator

<sup>9</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 237.

<sup>10</sup> Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 595.

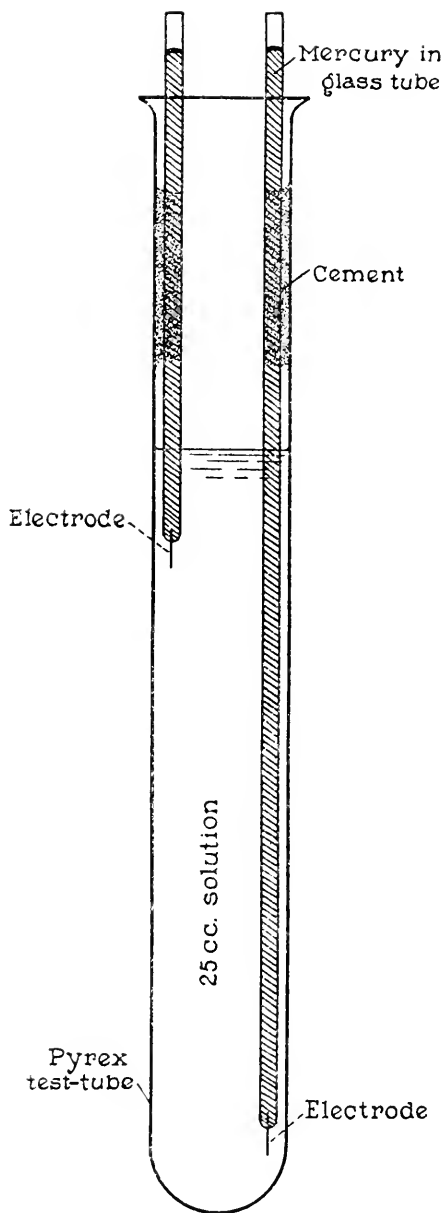


FIG. 1. Type of conductivity cell used. The cell consists of a Pyrex test-tube of about 40 cc. capacity and 20 mm. in diameter. The electrodes are made of No. 20 platinum wire sealed into fine Pyrex glass tubing. The electrodes are plated with platinum black and are 5 mm. long. The tubes holding the electrodes are fastened to the sides of the test-tube with de Kotynsky cement and are then filled with mercury. The distance between the electrodes is so adjusted that all the cells have the same constant; *i.e.*, when filled with the same solution they all read the same.

before adding the formaldehyde. The solution was then titrated to 8.2 with thymol blue. The titrations were made with 0.1 N NaOH. The end-points are both accurate to 0.05 cc.

*Technique of the Determination.*—The gelatin solution is melted, 25 cc. pipetted into a series of the conductivity cells and the cells suspended in the water bath at  $33^{\circ} \pm 0.01^{\circ}\text{C}$ . The conductivity is determined at intervals until it becomes constant (usually about 20 minutes). 1 cc. of the trypsin solution (previously warmed to  $33^{\circ}$  and having the same conductivity) is then added. The solution is then thoroughly mixed by sucking back and forth three times in a warm dry 15 cc. pipette. It is necessary to avoid air bubbles and to be sure that the solution is well mixed. Irregular results can nearly always be traced to incomplete mixing. The conductivity of the solution is then read at intervals so that readings are obtained at every 1.5 or 2 divisions on the bridge, until the reading is 485 or less (corresponding to a decrease of 15 points). The elapsed time is calculated from the time at which the trypsin is added. Since in order to obtain the elapsed time it is necessary to make a great many subtractions it is a great convenience to use a clock which is divided into hundredths of an hour instead of minutes. If the trypsin solution has been carefully adjusted to the same temperature and conductivity as the gelatin and the mixing carried out without change of temperature or the formation of air bubbles, it will be found that the readings form a perfectly smooth curve. It frequently happens, however, that the first reading (before the trypsin is added) does not fall on the same curve as the subsequent readings. In this case the curve is extrapolated back from the first reading after the trypsin is added in order to find the zero reading. Since, as was stated above, the curve is perfectly smooth when the experiment is done with sufficient care this procedure seems justified. The results should always fall on a smooth curve after the first 0.05 hour (corresponding to a change of from 0.1 to 1.0 on the bridge). The results are then plotted on a large scale and the time necessary for the reading to change 10 points determined by interpolation. This corresponds to an actual change in the conductivity of  $0.0782 \times 10^{-3}$  reciprocal ohms and is less than 10 per cent of the total change which can be effected by the trypsin under these conditions. Owing to the large number of experiments, the individual time curves from which the time necessary to cause the 10 points change is determined will not be given, but only the time interval interpolated from these curves. The points lie so close together on the curve that there is little or no possibility of arbitrary adjustment of the curve. The elapsed time is, therefore, a direct experimental determination. In the few cases where it was possible to draw more than one curve through the experimental points, the extreme values for the interpolated time have been given.

*Comparison of the Course of the Reaction as Followed by the Formal Titration of the Solution and by the Change in Conductivity.*

The most significant determination as regards the hydrolysis of proteins is the increase in amino or carboxyl groups. It was found that under the conditions adhered to in these experiments the increase in carboxyl groups is directly proportional to the increase in the conductivity. This is shown in Fig. 2. The values obtained by the conductivity method therefore represent the actual course of the hydrolysis. This is not true under all conditions. It was found that at other ranges of acidity the two determinations are not parallel. The change in conductivity is also dependent on the alkali used to bring the gelatin to the required pH. It is greatest in ammoniacal solution and may even decrease instead of increase in concentrated phosphate solutions.

Since the rate of hydrolysis is to be used to determine the amount of trypsin present it is necessary to have some method of expressing the velocity of the hydrolysis. This value should be independent of the stage of the hydrolysis at which the determination is made since otherwise it would evidently be possible to obtain a series of values depending on what stage of the hydrolysis was chosen. The most satisfactory figure for such purposes is of course the constant obtained by substituting the observed values in some equation such as the monomolecular reaction equation. The hydrolysis as carried out in these experiments, however, does not follow accurately any of the simple reaction formulæ so that this method cannot be used. It was found that the reciprocal of the time required to cause a definite change was very nearly directly proportional to the concentration of trypsin; *i.e.*,  $QT = K$ . Where  $Q$  is the concentration of trypsin,  $T$  the time required to cause a given small change, and  $K$  is a constant. As Arrhenius<sup>1</sup> has pointed out, this is a general property of enzymes even though they do not follow the formula for a monomolecular reaction. This rule has been found to hold for trypsin by Taylor,<sup>3</sup> Henri,<sup>8</sup> Vernon,<sup>11</sup> Hedin,<sup>12</sup> and Bayliss,<sup>7</sup> whereas Grützner<sup>13</sup> states that the rule does not hold, but that the reciprocal of the time

<sup>11</sup> Vernon, H. M., *J. Physiol.*, 1904, xxx, 330.

<sup>12</sup> Hedin, S. G., *J. Physiol.*, 1905, xxxii, 468.

<sup>13</sup> von Grützner, P., *Arch. ges. Physiol.*, 1911, cxli, 63.

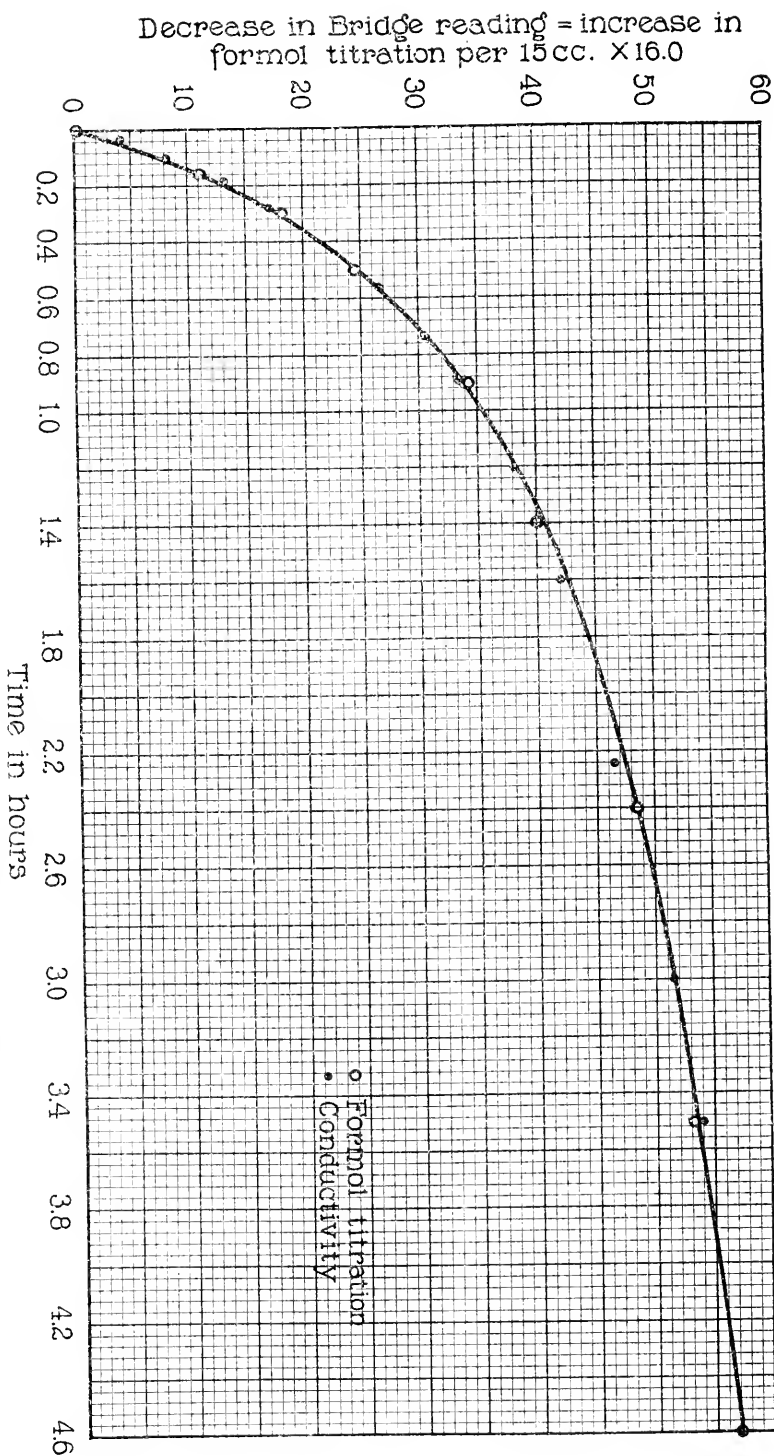


Fig. 2. Comparison of the increase in conductivity (as measured by the decrease in the bridge readings), with increase in the formol titration of the solution. The formol titration was made on 15 cc. of solution. The results have been plotted to the same scale by multiplying the formol titration by 16. The ordinates therefore represent the decrease in bridge reading or the formol titration (expressed as cc. 0.10 N NaOH) per 240 cc. of solution.



increases more rapidly than the amount of enzyme taken. It will be shown below that the results depend on the purity of the enzyme and protein solutions used. In the case of purified trypsin and

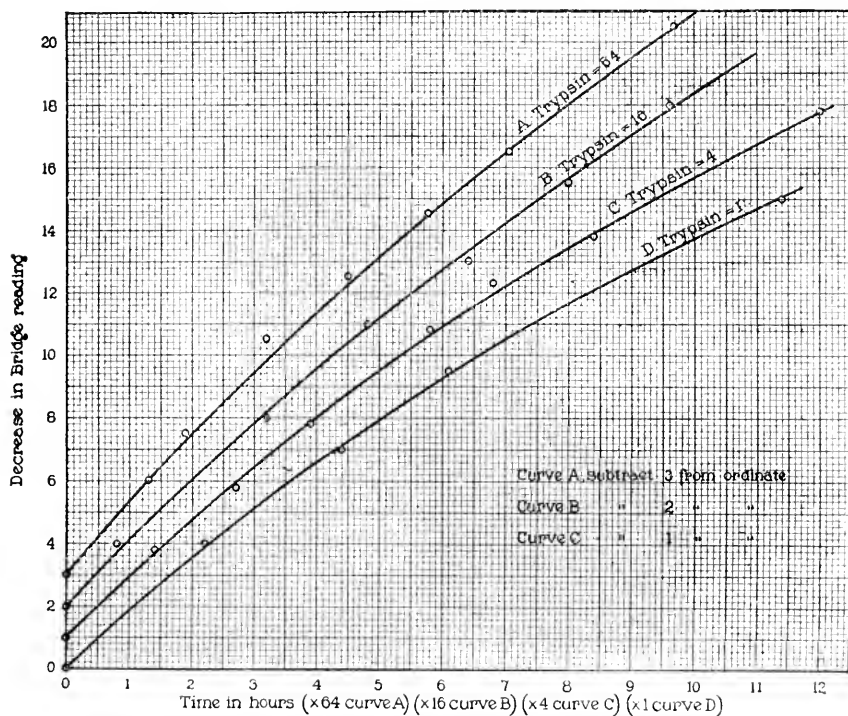


FIG. 3. Influence of the concentration of trypsin on the rate of digestion. 25 cc. gelatin plus 1,  $\frac{1}{4}$ ,  $\frac{1}{16}$ ,  $\frac{1}{64}$  cc. of trypsin solution. The change in the conductivity determined at intervals as shown in the figure. pH 6.4, temperature 33°. In order to give all the results in one figure the time units for each curve have been made proportional to the amount of trypsin added; *i.e.*, in the solution containing a relative trypsin concentration of 64 (1 cc.) the observed time has been multiplied by 64. To avoid confusion the ordinates (bridge readings) have been increased by 1 (Curve C), 2 (Curve B), and 3 (Curve A).

gelatin solutions the velocity (reciprocal of the time) is nearly directly proportional to the amount of enzyme taken within the limits that can be experimentally worked with. This fact is shown in Fig. 3. In plotting this figure the time units for each concentration of trypsin

have been multiplied by the relative concentration of trypsin used in obtaining the curve corresponding to that concentration. It will be seen that if the formula  $QT = K$  is correct, the resulting curves should be identical (or parallel if plotted as in the figure). As the figure shows this is not quite true. The curves for the concentrated solutions do not drop off as rapidly as those for the dilute. That is the

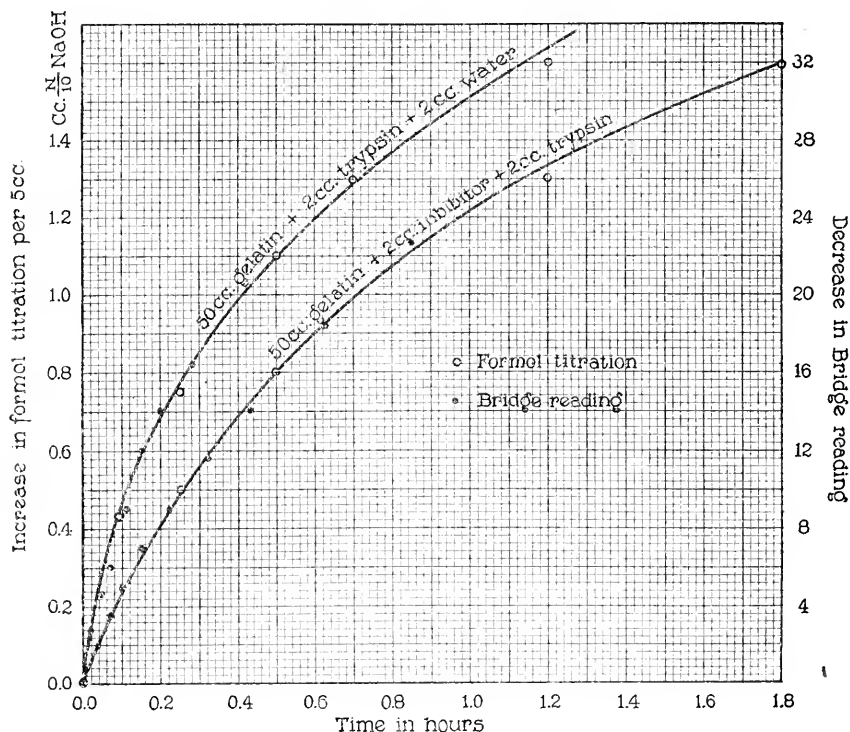


FIG. 4. Comparison of the retardation due to the presence of the "inhibitor" as determined by the formol titration or by the conductivity method.

velocity as measured by the reciprocal of the time increases more rapidly than the trypsin concentration. This is due, as will be shown later, to two causes. First, the products formed by the action of the trypsin inhibit its action, and since at any given stage of the hydrolysis the concentration of the products in the different solutions will be the same but the concentration of trypsin different, the percentage

inhibition of the solution containing a small amount of trypsin will be greater than in that containing a larger amount of trypsin. Second the trypsin is constantly becoming irreversibly inactivated. The amount of this inactivation is proportional to the time and is therefore proportionately greater in the dilute solutions since these require a longer time to cause the change used as the end-point. This effect is much more noticeable at a higher temperature, as would be expected.

TABLE I.

Relative concentration of trypsin.	Time required to change 10 points observed.	( $QT = 550$ ) calculated.	$QT$
	<i>hrs. <math>\times 10^2</math></i>		
64	7.5	8.5	480
	7.3		467
32	17.5	17.0	560
	16.0		510
16	34	34.0	540
	35		560
8	80	68	640
	82		656
4	155	136	620
	160		640
2	300	272	600
	250		500
1	660	542	660
	680		680

Both these effects are in the same direction and both become more noticeable the longer the hydrolysis proceeds, as Bayliss<sup>7</sup> found. The experiment was made in duplicate and was run with intermediate trypsin concentrations which are not plotted. Table I gives a summary of the whole experiment. The table shows that the values found for the amount of trypsin are within about 10 per cent of the expected values over the entire range of the experiment and much closer for smaller variations of the trypsin concentration. This dif-

ference is not much greater than the experimental error. The experiment was repeated several times and it was found that in general the reciprocal of the time required to cause a change of 10 points in the bridge reading was directly proportional, within the experimental error, to the amount of trypsin taken. This value has therefore been used to express the amount of active trypsin present in the solution. The unit of trypsin used in these experiments may be defined as that quantity which when added to 25 cc. of gelatin solution having a pH of 6.3 (adjusted + NaOH) and a specific conductivity of  $2 \times 10^{-3}$  (adjusted + KCl) will cause a change in the bridge reading of 10 points (500 - 490) in 1 hour at 33°C. (equivalent to an increase in the conductivity of  $0.0782 \times 10^{-3}$  reciprocal ohms).

#### *Properties of the Trypsin Used.*

It has been stated by Vernon<sup>11</sup> and others that "trypsin" may be separated more or less into a number of enzymes some of which attack gelatin more rapidly than other proteins and some of which act best on peptones. If this is the case the experiments are evidently complicated by another factor in addition to the many already present. Many experiments were made but no evidence could be found to show the presence of such enzymes in the sample of trypsin used in these experiments. The trypsin was treated in a number of different ways and the relative velocity with which it hydrolyzed gelatin or peptone compared before and after the treatment. (The peptone was prepared by the action of pepsin on gelatin sulfate solution. The acid was then removed with barium and the solution made alkaline with NaOH.) A summary of these experiments is given in Table II. It will be seen that there is no evidence for the existence of any special "peptonase" or "gelatinase" in the sample of trypsin used in these experiments.

#### *Effect of Dialysis on the Trypsin.*

It has already been stated that the trypsin was purified by dialysis. The effect of this is shown in Table III. It will be seen that the activity of the trypsin is more than doubled by the process and that the solution obtained in this way contained only about 0.02 gm. per cc. of total solids.

TABLE II.

*Influence of Treatment of Trypsin on Ratio of Peptonase and Protease.*

Gelatin, 2 per cent, pH 6.2, specific conductivity 0.0022. Gelatin peptone, gelatin hydrolyzed by trypsin, 2 per cent, pH 6.2, specific conductivity 0.0030.

Treatment of trypsin.	$T_0 - 10$ gelatin.	$T_0 - 10$ gelatin peptone.	$T_0 - 10$ gelatin pep- tone. $T_0 - 10$ gelatin
	hrs. $\times 10^2$	hrs. $\times 10^2$	
1 per cent Fairchild's trypsin solution.	25	22	1.14
	29	24	1.21
	26	24	1.09
	28	28	1.00
	34	32	1.07
	35	30	1.17
Average.....			1.11
10 gm. trypsin dissolved in 100 cc. water, pre- cipitated with 50 cc. alcohol. Precipitate = 0.1 gm. dissolved in 20 cc. H <sub>2</sub> O.	30	24	1.24
	27	22	1.22
	29	28	1.04
	32	31	1.04
	37	31	1.19
Average.....			1.14
50 cc. 10 per cent trypsin dialyzed 18 hours at 6°C., brought to pH 6.8 + NaOH, then left 3 hours at 38°C. (about 98 per cent inacti- vated).	28	27	1.0
	25	33	0.7
	23	32	0.7
Average.....			0.8
Control.	40	46	0.86
	35	36	0.90
	37	36	0.90
Average.....			0.88
50 cc. 10 per cent trypsin dialyzed 18 hours at 6°C., brought to pH 4.2 with HCl and kept at 38°C. for 1 hour, about 80 per cent inacti- vated.	25	22	1.13
	25	21	1.20
	26	26	1.00
	24	24	1.00
Average.....			1.08
Control.	39	33	1.18
	48(?)	37	1.27
	50	44	1.10
	45	46	0.98
Average.....			1.13

TABLE III.

*Dialysis of Trypsin.*

2.5 gm. of trypsin in 25 cc. water, dialyzed under pressure 18 hours, and filtered.

	Time required to change 10 points.	Trypsin per cc.	Dry weight 1 cc.	Trypsin per gram.	Formol titration.	pH
	<i>hrs. × 10<sup>2</sup></i>	<i>units</i>	<i>gm.</i>	<i>units</i>	<i>cc. 0.1 N NaOH</i>	
Before dialysis.....	5.0	20	0.10	200	3.3	
After dialysis.....	10.0	10	0.02	500	0.4	6.2 to 6.4

*Preparation of the Inhibiting Solution.*

Bayliss<sup>7</sup> found that glycine and other amino-acids as well as the digested protein solution inhibited the action of trypsin. Several amino-acids were tried but with negative results except in concentration very much higher than could possibly be present from the protein. In such high concentration the determination becomes uncertain, owing to the high conductivity of the solution. Bayliss' experiments were made at a time when the determination of the hydrogen ion concentration was a difficult matter and it seems possible that the results he obtained were due to changes in the pH caused by the addition of the amino-acids rather than to an effect on the enzyme. A solution of casein or gelatin which has been hydrolyzed by trypsin does show marked inhibitory effects, however. Owing to the manner in which these experiments were made it was necessary to have the solution nearly salt-free and in a concentrated form so that the volume change on adding it to the gelatin would be small. It is also necessary to be sure that there are no products left in solution that can be further acted on by trypsin. It was found that a solution having strong inhibitory powers could be made from either gelatin or casein by the following method.

*Preparation from Gelatin.*—3 liters of 1.5 per cent gelatin were titrated to a pH of 9.0 with Ba(OH)<sup>2</sup> and 10 cc. dialyzed trypsin and a few crystals of thymol added. The solution was kept at 23° until no further increase in the formol titration could be noted. The titration increases about 700 per cent. The solution was then titrated to a pH of 6.3 with sulfuric acid and the barium sulfate

filtered off. The solution was then evaporated in vacuum to 60 cc. 1 cc. of this solution contained the equivalent of 0.7 gm. of gelatin and had a formol titration of 16 cc. of 0.1 N NaOH.

*Preparation from Casein.*—200 gm. of commercial casein were dissolved in 3 liters of water and precipitated by the addition of sulfuric acid. The precipitate was washed in water, suspended in 1 liter of water and heated to boiling.  $\text{Ba}(\text{OH})_2$  was then added until the supernatant liquid had a pH of 9.0. The solution was cooled and 5 cc. dialyzed trypsin added and the solution kept at 23° for 10 days. It was then filtered, the filtrate titrated to pH 6.3 with sulfuric acid, evaporated in vacuum to 100 cc., and precipitated by the addition of 800 cc. of 95 per cent alcohol. A gummy precipitate forms which consists of higher products which are still acted upon by trypsin. The filtrate is evaporated *in vacuo* to a thick syrup to remove the alcohol and taken up in 50 cc. water. The solution so obtained is a clear yellowish syrup. It is not further acted upon by trypsin and contains no active trypsin. The formol titration per cubic centimeter is equivalent to 15 cc. of 0.1 N NaOH. This solution was then accurately adjusted to pH 6.3 with HCl and to a specific conductivity of  $2 \times 10^{-3}$  reciprocal ohms by the addition of a small amount of KCl. The experiments described in this paper were all made with the solution prepared in this way from casein. It is referred to as inhibiting solution or inhibitor.

#### *Properties of the Inhibiting Solution.*

It had already been stated that no effect could be noted if amino-acids were added to the trypsin, unless very much higher concentrations were used than could be furnished by digestion of the protein.

Glycine, alanine, tryptophane, leucine, tyrosine, arginine, proline, and histidine were tested alone and in combination. It was also found that gelatin or casein which had been completely hydrolyzed by either acid or alkali was without effect. The results of some of these experiments are shown in Table IV. The experiment shows that the inhibiting substance is not formed in the acid or alkali hydrolysis of gelatin or casein, and that it is dialyzable. The evidence is not sufficient to prove that the inhibiting substance is a specific result of trypsin hydrolysis since the acid and alkali hydrolyses were only tested when the reaction had continued far beyond the stage reached by trypsin hydrolysis. It may be mentioned, however, that it has been found by an entirely different method that the hydrolysis of gelatin by acids or alkalies or trypsin in the early stages follows a different course and must give rise to different products in each

case.<sup>14</sup> The action of pepsin gives rise to substances which inhibit pepsin but which are themselves attacked by trypsin.

TABLE IV.

*Retarding Effect of Various Solutions of Hydrolyzed Gelatin or Casein on the Action of Trypsin.*

25 cc. of gelatin pH 6.2, specific conductivity  $2 \times 10^{-3}$ , plus 1 cc. of solution noted. 1 cc. of trypsin added and time required to cause a change of 10 points (bridge reading) in the conductivity determined. In each case the amount of hydrolyzed protein added is equivalent to 0.2 gm. of the original protein.

Solution.	Time required to change 10 points.	
	Control.	+ 1 cc. solution.
	hrs. $\times 10^2$	hrs. $\times 10^2$
(a) Gelatin hydrolyzed by trypsin.....	22	45
(b) Casein hydrolyzed by trypsin.....	15	36
(c) Casein completely hydrolyzed	by acid.....	25
	by alkali.....	40
		27
(d) Gelatin completely hydrolyzed	by acid.....	17
	by alkali.....	19
		34
		30
(e) Solution (a) after 24 hrs. dialysis.....	16	15
	14	15
Solution (b) after 24 hrs. dialysis.....	28	29
	24	26

*Method of Determining the Amount of Retardation.*

It has already been shown that the rate of hydrolysis of gelatin alone as determined by the change in conductivity is identical with that found by the formol titration. Fig. 4 shows that this is also the case when the hydrolysis has been retarded by the addition of inhibitor

<sup>14</sup> Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 57.



solution. The figure and Table V show that the retardation is nearly independent of the stage of hydrolysis compared for the first part of the reaction but then becomes relatively less. The retardation may therefore be calculated from the velocity of the reaction provided the early part of the reaction curves are compared. (It has been found by Simons<sup>15</sup> in Nelson's laboratory that this is not the case with invertase.) The result found with trypsin, namely, that the retarding effect of the inhibiting solution becomes less as the reaction proceeds, is exactly what would be expected if it is supposed that the inhibitor combines with the enzyme to form an inactive com-

TABLE V.

*Influence of Inhibitor on Rate of Hydrolysis Followed by Formol Titration and by Conductivity.*

Temperature, 33°C. 2.0 per cent gelatin, pH 6.2 + NaOH. Specific conductivity,  $2.2 \times 10^{-3}$ .

Increase in formol titration per 5 cc. solution. (a)	Decrease in bridge reading.	Time required to cause increase in formol titration given under (a).		
		Control.	Inhibitor.	$\frac{T_{\text{inhibitor}}}{T_{\text{control}}}$
cc. 0.1 N NaOH	units	hrs.	hrs.	
0.3	6	0.06	0.13	2.17
0.5	10	0.12	0.26	2.16
1.0	20	0.41	0.72	1.70
1.5	30	1.00	1.54	1.54

pound and that more of the inhibiting substance is formed during the hydrolysis (or some of the trypsin destroyed). The retarding effect of the inhibiting substance formed during the reaction will evidently be much less in the solution that already contained the inhibitor than in the solution which contained only free trypsin. The inhibitor acts just as a "buffer" solution for regulating the hydrogen ion concentration, except that in this case it is the enzyme that is "buffered." In fact the same experiment may be performed by following the hydrolysis of gelatin with a weak as compared with a

<sup>15</sup> Simons, L. S., Dissertation, Columbia University, 1921.

strong acid at about the same pH.<sup>16</sup> This mechanism will evidently lead to the result that the solution containing inhibitor will be relatively more active compared to the control solution as the hydrolysis proceeds, which is the experimental fact. The same fact has been noted in immunology in comparing the action of free toxin and of a mixture of partially neutralized toxin. Bordet<sup>17</sup> considers that it is the degree of activity of the total amount of toxin that is affected by the antitoxin and not the concentration of free toxin. His experiment is similar to the trypsin experiment just discussed, inasmuch as he found that a small amount of free toxin reacts at first more rapidly than a mixture of toxin-antitoxin, but that as the reaction proceeds, the mixture becomes relatively much more efficient. Bordet's explanation will not explain the results with trypsin quantitatively while the assumption of the formation of an inactive compound between the trypsin and inhibitor allows all the peculiarities of the reaction to be calculated. It does not follow, of course, that the toxin-antitoxin reaction is the same as the trypsin-inhibitor reaction, but it seems that the same explanation will apply qualitatively to both.

<sup>16</sup> If the pH of the solution containing the strong acid is slightly lower than that of the weak acid, the rate of hydrolysis will at first be greater in the solution containing the strong acid. The rate of hydrolysis in this solution will decrease rapidly, however, since the concentration of hydrogen ions will be diminished by the products of hydrolysis—just as is the concentration of free trypsin in the present experiment. The rate of hydrolysis in the weak acid solution, however, will remain nearly constant since the hydrogen ions which combine with the products formed will be replaced by the dissociation of more of the weak acid. The rate of hydrolysis in the weak acid solution will therefore constantly increase as compared to the rate of hydrolysis in the strong acid solution. An example of such an experiment is given in a preceding paper (Northrop, J. H., *J. Gen. Physiol.*, 1920-21, iii, 725, Fig. 3, Curves III and IV).

<sup>17</sup> Bordet, J., *Immunité*, Paris, 1920, p. 530.

## THE INACTIVATION OF TRYPSIN.

### II. THE EQUILIBRIUM BETWEEN TRYPSIN AND THE INHIBITING SUBSTANCE FORMED BY ITS ACTION ON PROTEINS.

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#### *The Equilibrium between Trypsin and the Inhibiting Substance.*

The experiments already described<sup>1</sup> show that it is possible to prepare a solution by the action of trypsin on a protein which inhibits the action of trypsin. It has also been shown that the amount of this retardation can be quantitatively measured by comparing the times necessary to cause a given small change in the conductivity of the gelatin solution under the conditions adhered to.

A number of hypotheses may be proposed that will account qualitatively for this retardation. The simplest would be to assume that the inhibiting substance combined with trypsin to form a compound that is inactive and that the activity of the solution is proportional to the concentration of free trypsin remaining in the solution. It has already been shown that if pure trypsin and protein is used the velocity of hydrolysis is proportional to the amount of trypsin taken. This is the experimental fact and is independent of any hypothesis as to the kinetics of the reaction. If it is further assumed that the equilibrium is governed by the law of mass action it is possible to test this hypothesis quantitatively. This has been done in the following experiments.

#### *Influence of the Order of Mixing and of the Time of Standing on the Equilibrium.*

Since in most of the experiments the retarding effect of the inhibiting solution has been determined by adding the solution to the gelatin

<sup>1</sup> Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 227.

and then adding the trypsin and determining the rate of hydrolysis at once, it is necessary to know whether or not the order of mixing the solutions or the time during which the trypsin has been allowed to react with the inhibiting substance has any influence on the result. Table I is a summary of experiments planned to answer this question. It shows that the order of mixing and the time during which the trypsin and inhibiting solution are left together has no effect on the final result. That is, the equilibrium between the trypsin and the inhibitor must be reached practically instantaneously and be quantitatively and instantly reversible. (This is only true if the experiment

TABLE I.  
*Effect of Order of Mixing and Time of Standing.*

SOLUTION.	Time to change 10 points after hours at 6°C.	
	0 hours.	1.0 hours.
	hrs. $\times 10^2$	hrs. $\times 10^2$
(1) 25 cc. gelatin + 1 cc. trypsin.....	15 16	16
(2) 25 cc. gelatin + 1 cc. inhibitor + 1 cc. trypsin added after interval shown.....	21 25	21 20
(3) 25 cc. gelatin + 2 cc. mixture of 5 cc. inhibitor + 5 cc. trypsin. (Mixture allowed to stand as noted and 2 cc. then added to gelatin).....	22	20

is made under such conditions that the control trypsin solution—without inhibitor—remains unchanged during the course of the experiment. If this condition is not fulfilled, the results depend entirely on the length of time and on the temperature at which the trypsin-inhibitor solution has been allowed to stand.)<sup>2</sup>

*Influence of the Gelatin Concentration.*

The retardation could be qualitatively accounted for by assuming that the inhibiting substance combines with the gelatin instead of

<sup>2</sup> Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 261.

with the enzyme. If this were true the retardation should be less the greater the concentration of gelatin, provided the relative amount of inhibitor to trypsin were kept the same. Table II shows that this is not the case. The amount of retardation is independent of the concentration of gelatin used. The result is confirmed by the experiment discussed below in which the concentration of inhibitor is kept the same and the concentration of trypsin varied. If the inhibitor combined with the gelatin, the resulting solution would act as though

TABLE II.

*Influence of Gelatin Concentration on Retardation of Hydrolysis by Inhibiting Solutions.*

*Control*, 25 cc. gelatin of concentration noted + 1 cc. trypsin + 1 cc. 0.01 N NaCl. *Solution*, 25 cc. gelatin of concentration noted + 1 cc. trypsin + 1 cc. inhibiting solution. Temperature 33°C. Specific conductivity of all solutions  $1.2 \times 10^{-3}$  (adjusted with NaCl, and pH of 6.0 adjusted with NaOH).

Time required for 10 points change with 1 cc. trypsin Time required for 10 points change with 1 cc. trypsin + 1 cc. inhibitor in gelatin concentrations of			
1 per cent.	2 per cent.	4 per cent.	8 per cent.
66	65	67	
67	65		
70	70		73
75	75	75	76
76	70	73	71
	75	73	72
Average $70.8 \pm 1.8^*$	$70.0 \pm 1.4^*$	$72.0 \pm 1.2^*$	$73.0 \pm 0.7^*$

\* Average deviation of the mean.

a lower concentration of gelatin had been used and the velocity would still be directly proportional to the amount of trypsin added to the solution. This is not the case. The fact that the inhibiting solution renders trypsin more stable (when no protein is present) also shows that it combines with the trypsin.

#### *Influence of Inactivated Trypsin.*

It was found in the case of pepsin<sup>3</sup> that pepsin inactivated by alkali took part in the equilibrium just as does the active pepsin

<sup>3</sup> Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 471.

(pepsin inactivated by heat does not act in this way). If it should be found that inactive trypsin also took part in the equilibrium it is evident that the experiments are complicated by an additional factor that is very hard to control since there is no independent method for determining the amount of inactive trypsin. In order to determine this point the experiments described in Table III were performed. They show that the inactive trypsin does not take part in the equilibrium. The calculated results are obtained from the law of mass action as described below.

TABLE III.

*Addition of Inactive Trypsin.*

2 per cent gelatin pH 6.2, specific conductivity  $2 \times 10^{-3}$ . Trypsin, 10 per cent, dialyzed, time to change 10 points = 0.10 hours = 10 units per cc. Inactivated at 65°C. for 2 hours. Active trypsin, 10 per cent, dialyzed, diluted one-third.  $P = 10$ .  $K' = 2.8$ .

Active trypsin.	Inhibitor.	Inactive trypsin.	Time to change 10 points.	Q		
				Observed.	Calculated.	
					Inactive no effect	Inactive enters equilibrium.
cc.	cc.	cc.	hrs. $\times 10^2$			
1	0	0	36 39	2.7	2.7	
1	0.5	0	90 87	1.1	1.2	1.2
1	0.5	1	90 80 95	1.1 1.2 1.05	1.2	1.9

*Effect of Adding Increasing Amounts of Inhibitor to a Constant Quantity of Trypsin.*

The results of a series of experiments to determine the effect of adding increasing concentrations of inhibitor to the same amount of trypsin are given in Table IV and Fig. I. The experiments were carried out by adding the noted amount of inhibiting solution to 25 cc. of gelatin at 33°C. All the solutions had the same pH, which re-

TABLE IV.

*Effect of Increasing Amounts of Inhibitor on the Rate of Hydrolysis.*

25 cc. gelatin pH 6.2; specific conductivity  $2 \times 10^{-3}$ . 1 cc. trypsin (10 per cent dialyzed).  $V = 28$  cc.  $P = 10$ .  $K' = 2.8$ .

Cc. inhibitor $= \frac{d}{10}$	Time to change 10 points.  <i>hrs. <math>\times 10^2</math></i>	Trypsin.			$\frac{Qd}{10}$	Trypsin combined per 0.125 cc. of inhibitor added
		Free $\frac{1}{T} = Q$ observed.	Combined. $E - Q$ observed.	Calculated. $Q$		
0	44 48	2.3	0	2.3		
0.125	52	1.9	0.4	1.81	0.24	1st 0.125 cc. 0.40
0.25	64	1.56	0.74	1.45	0.39	2nd " 0.34
0.50	90	1.10	1.20	1.00	0.55	3+4th " 0.18
1.00	155	0.65	1.65	0.58	0.65	5 to 8th " 0.11
2.00	300	0.33	1.97	0.32	0.66	9 to 16th " 0.03

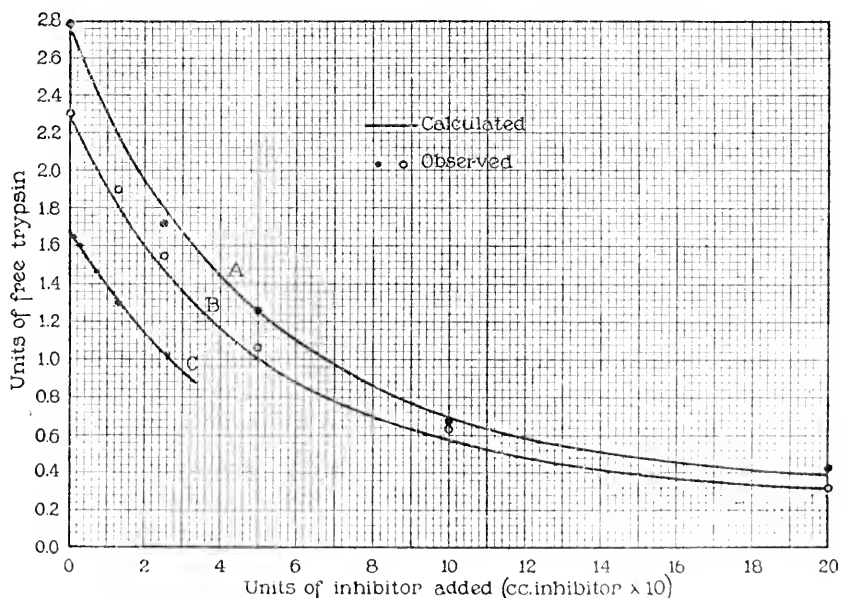
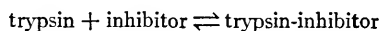


FIG. 1. Effect of adding increasing amounts of inhibitor to trypsin solutions of different strengths. The solid curves are the calculated values, and the points, the observed units of active trypsin present. (This is taken as the reciprocal of the time in hours necessary to cause a change in the bridge reading of 10 points.)

mained constant at 6.3, and the same initial conductivity. 1 cc. of trypsin solution, also of the same pH and conductivity, was then added in each tube and the time required to cause a change of 10 points in the bridge reading determined as already described. The experiments show that the addition of the first cubic centimeter of inhibiting solution has a much greater effect than the subsequent ones and that the effect constantly diminishes and becomes apparently asymptotic. This is evidently the cause of the phenomenon noted earlier that the rate of hydrolysis of a solution already containing some inhibiting substance is less rapidly retarded during the progress of the reaction than one which contains only "pure" trypsin. This is in qualitative agreement with the result predicted by the law of mass action. In order to apply this law quantitatively we may proceed as follows. It has been assumed that the equilibrium was expressed by the equation



and that the rate of hydrolysis was proportional to the concentration of the free trypsin. The law of mass action applied to this equilibrium states that

$$\frac{\text{Concentration of free trypsin} \times \text{Concentration of free inhibitor}}{\text{Concentration of trypsin-inhibitor}} = \text{a constant}$$

or

$$\frac{\frac{Q}{V} \cdot \left[ \frac{d - (E - Q)}{V} \right]}{\frac{E - Q}{V}} = K$$

which may be written

$$\frac{Q [d - (E - Q)]}{(E - Q)} = KV = K' \quad (1)$$

in which  $Q$  is the amount of free trypsin in volume,  $V$ , of the solution;  $E$ , the total amount of trypsin in volume,  $V$ , of the solution;  $d$ , the total amount of inhibitor in volume,  $V$ , of the solution;  $K$ , the equilibrium constant in arbitrary units; and  $K'$ , new constant equal to the product of the equilibrium into the volume.  $d$  will evidently be proportional to the number of cc. of inhibiting solution added and if there are  $P$  units of inhibitor per cc. of inhibiting solution,  $d = P$  cc.



Solving this equation for  $Q$  we find that

$$Q = \pm \sqrt{\left(\frac{d - E + K}{2}\right)^2 + KE} - \frac{d - E + K}{2}$$

and since it is assumed that the velocity of the reaction is proportional to  $Q$

$$\frac{dx}{dt} \propto \frac{\Delta X}{\Delta T} \propto \frac{I}{T} \propto Q \quad \text{and} \quad \frac{T_1}{T_2} \propto \frac{Q_2}{Q_1}$$

All the values in the above equations are known (in arbitrary units) except  $d$  and  $K$ . If more than one experiment is made it is therefore possible to solve for these two values, and then compare the calculated and observed values for  $Q$ . When this is done it is found that below a certain value for  $d$ ,  $K$  is negative while above a certain limit the value of  $\frac{K}{d}$  becomes constant owing to the fact that one of the terms of the equation becomes negligible when  $d$  is too large. It is also found that the constancy of  $K$  is very sensitive to experimental error (as was to be expected since it depends on the difference between two experimental values), so that a comparison of the observed and calculated values of  $Q$  is a better test of the formula than the constancy of  $K$ . Between the two limits for the values of  $d$  there are several values all of which give values of  $K$  which permit the calculation of the experimental results. The smallest of these has been taken. This gives a value for  $K$  of 0.1 and for  $P$  (in the particular inhibiting solution studied) of 10, both expressed in the same arbitrary units (*cf.* Euler and Svanberg<sup>4</sup> for a discussion of the same equilibrium with invertase, and Northrop<sup>3</sup> in the case of pepsin). It will be noted that the expression for the equilibrium as used in this form contains two arbitrary constants; *i.e.*, it is necessary to make two determinations before the others can be calculated. The agreement between the calculated and observed values is close enough to leave little doubt that the formula correctly expresses the facts, but the presence of two constants renders it possible that the agreement is accidental. If this were the case we should expect to find that it was necessary to use different values for  $K$  and  $d$  in each set of experiments. This is, however, not the case. All the experiments were found to agree

<sup>4</sup> von Euler, H., and Svanberg, O., *Fermentforschung*, 1920, iii, 330; 1921, iv, 142.

with a value for  $K$  of 0.1 as found above, and all done with the same inhibiting solution to agree with the same value of  $P$  (or  $d$ ) as well. Several experiments were made which gave apparently regular results for which, however, it was impossible to find any satisfactory value for  $K$  and  $d$ . In every case of this kind it was found that the inhibiting solution used contained either active trypsin or some substance which could still be acted on by trypsin. In applying the formula, it is assumed, of course, that the only trypsin present is that in the trypsin solution added and that the substrate concentration is the same in all tubes. These experiments seem to show that the formula is not of such a general character as to fit any regular curve. As would be expected, different inhibiting solutions required different values of  $P$ . The results shown in the figure were all calculated from the same values of  $K'$  which were obtained from the first part of the experiment in Curve A. All the other results were calculated before the experiment was done, as were those described later in which the conditions were varied in other respects. The figure shows that the calculated and experimental results are identical.

Column 6 in Table IV contains the values for  $Qd$ ; *i.e.*, the product of the free trypsin into the total amount of inhibitor. It will be seen that this value increases at first but becomes constant as  $d$  becomes large with respect to  $Q$ .  $Q$  therefore becomes inversely proportional to  $d$ . This may also be predicted from the mass action expression, and, as Arrhenius<sup>5</sup> pointed out, is the condition that leads to Schütz's rule. The steps in the derivation are as follows:

Equation (1) may be written

$$Q = \frac{K(E - Q)}{d - (E - Q)}$$

as  $d$  increases the term  $(E - Q)$  increases and approaches the constant value  $E$  so that  $K(E - Q)$  approaches a constant value. If  $d$  is large compared to  $E$  the term  $(E - Q)$  may be neglected in the denominator and the equation written

$$Q = \frac{K''}{d}$$

or  $Q$ , the amount of active trypsin is inversely proportional to the amount of inhibitor, which is the experimental result referred to

<sup>5</sup> Arrhenius, S., *Medd. Kong. Vetsakad. Nobelinst.*, 1908, i.

above (therefore  $Qd$  becomes constant). Since the velocity of hydrolysis is proportional to  $Q$  and to the concentration of substrate the differential expression for the course of hydrolysis would be  $\frac{dx}{dt} = QA$ , where  $A$  is the amount of substrate. Substituting  $\frac{K''}{d}$  for  $Q$  we have

$$\frac{dx}{dt} = \frac{K'' A}{d} \quad (2)$$

Since  $d$ , the inhibiting substance, is the same as  $x$ , the products of hydrolysis, we may substitute  $x$  for  $d$ .  $A$  may be considered a constant for the first few per cents of the hydrolysis. In any case, although  $A$  is decreasing, the term  $(E - Q)$  (which has also been considered a constant in the numerator) is increasing so that the product of the two will be more nearly constant than either of the two quantities themselves. It is this product that really enters into the equation. Substituting  $x$  for  $d$  in equation (2) and integrating we obtain

$$\int x \, dx = \int K'' A \, dt \text{ or } \frac{1}{2}x^2 = K_3 T \text{ or } x = K_4 \sqrt{T}$$

That is,  $x$ , the products formed, is proportional to the square root of the elapsed time. This derivation makes it clear that Schütz's rule will only hold when the concentration of the products formed is large with respect to the amount of enzyme. The author has shown that the same experiments may be performed with pepsin.<sup>3</sup> In the case of these two enzymes, at least, therefore, there is direct experimental evidence for Arrhenius' explanation.

*Effect of Constant Quantity of Inhibitor on Increasing Amounts of Trypsin.*

In all the foregoing experiments the concentration of trypsin has been the same in any one series of experiments and the concentration of inhibitor varied. If the mechanism proposed is correct it should be possible to predict equally well the result of an experiment in which the concentration of inhibitor was kept constant and the amount of trypsin varied. That this is the case is shown in Fig. 2. The calculated results for this experiment were obtained by using the

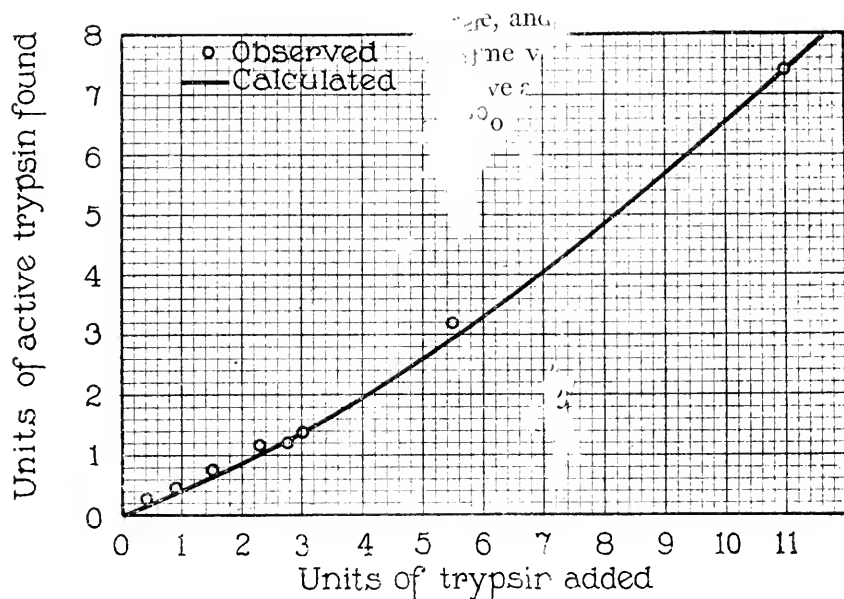


FIG. 2. The influence of the total amount of trypsin on the inactivation caused by 5 units inhibitor. Increased amounts of trypsin were added to series of tubes each containing 25 cc. gelatin solution, and 5 units inhibitor. Duplicate series run at the same time and under the same conditions, but without inhibitor.

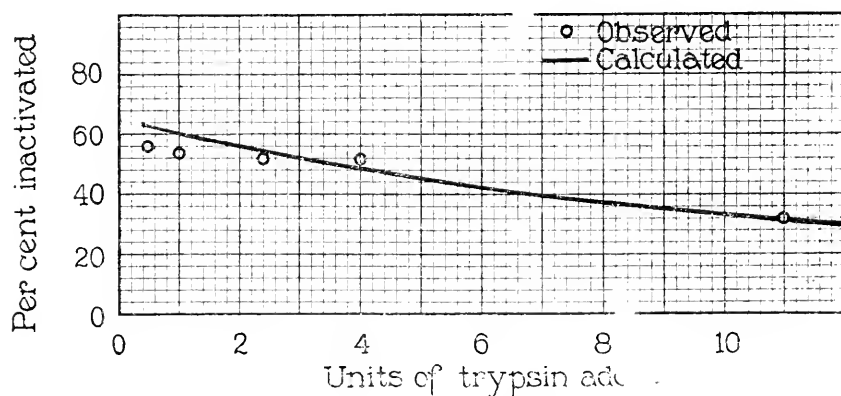


FIG. 3. The influence of the total amount of trypsin present on the percentage retardation caused by 5 units of inhibitor.

values for the constants  $k_1$  and  $k_2$  from the preceding experiments and were worked out before the experiment itself was done. The figure shows that in this case the experimental and calculated results agree within the limits of experimental error. In Fig. 3 the percentage inactivation of various amounts of trypsin by the five units of inhibitor have been plotted. As would be expected the smaller the amount of trypsin the greater the percentage inactivation although the absolute amount of trypsin inactivated is less.

It has been shown above that the law of mass action predicts quantitatively the results of the experiments when either the trypsin or the inhibitor concentration is varied. It is possible to vary conditions in another way by keeping the relative amount of trypsin and inhibitor the same and varying the dilution (*i.e.*, the value of  $v$ ). The calculated and observed results of such an experiment are given in Fig. 4 (Curve B). The experiment was performed by mixing the trypsin and inhibitor solution and then adding the noted cubic centimeters of this mixture to 25 cc. of gelatin. It will be seen that in this case also the predicted results are in close agreement with the experiment. In this case the rate of hydrolysis decreases more slowly than the total amount of trypsin taken. This is the result of the fact that as the dilution is increased the trypsin inhibitor compound dissociates and so liberates more active trypsin, so that the concentration of active trypsin does not decrease directly as the total trypsin. Exactly the same curve would be obtained for the rate of hydrolysis by hydrogen ions furnished by a weak acid if the total concentration of acid were plotted against the rate of hydrolysis. In Curve C in Fig. 4 the result of an experiment is given in which the concentration of trypsin is varied but the concentration of inhibitor is kept constant. This is a similar experiment to that described in Fig. 2. In this case the rate of hydrolysis decreases more rapidly than the concentration of the trypsin. This is the result of the fact shown in Fig. 3 that the percentage retardation of the action of trypsin with a constant concentration of inhibitor is the greater, the smaller the total amount of trypsin. Curve A in Fig. 4 is the dilution-activity curve for "pure" trypsin and gelatin. In this case the velocity is nearly directly proportional to the amount of trypsin taken. It is clear from these curves that unless care is taken to purify the

enzyme and protein solution used, activity-concentration curves may be found to be either convex or concave or a straight line. This probably accounts for the discrepancies in the literature in regard to this point. If the enzyme solution contained products of protein digestion, as is very likely to be the case, the rate of hydrolysis would not increase as rapidly as the enzyme concentration. If the protein solution was already partially hydrolyzed or contained some inhibiting substance, the velocity of hydrolysis would increase more rapidly than the enzyme concentration.

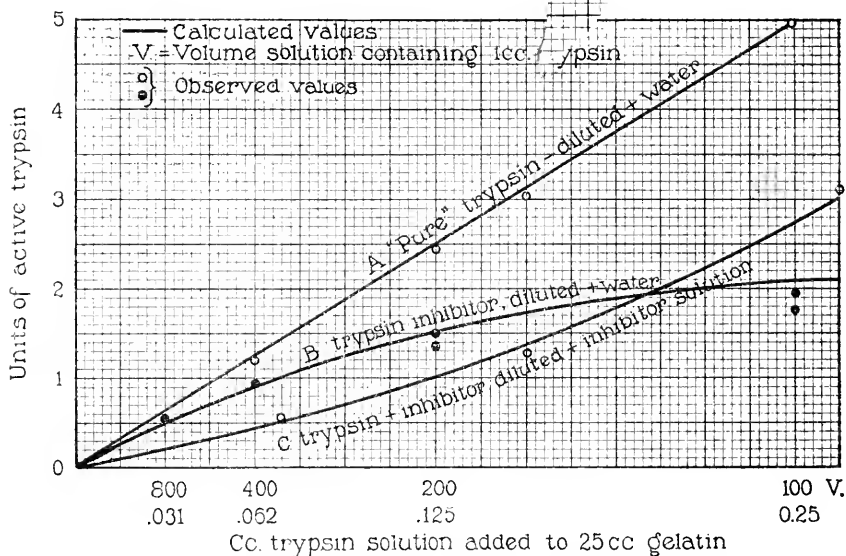


FIG. 4. The influence of the presence of inhibitor on the concentration-activity curve of trypsin. Curve A, "pure" trypsin diluted with water. Curve B, mixture of trypsin and inhibitor diluted with water. The ratio of trypsin to inhibitor is therefore constant. Curve C, mixture of trypsin and inhibitor diluted with a solution of inhibitor of the same concentration as was present in the trypsin solution. The concentration of inhibitor is therefore constant in this experiment.

#### *Effect of the Hydrogen Ion Concentration.*

In all the foregoing experiments the hydrogen ion concentration was kept constant at a pH of 6.3. It seemed of interest to determine what effect a variation in this factor would have on the retarding

action of the inhibiting solution. This experiment is difficult to perform since the conductivity method cannot be used and the pH can only be kept constant at ranges other than pH 6.3 by the use of high concentration of buffers which interfere with the formol titration. The results of such an experiment are given in Table V. The pH of the solutions was adjusted by making them all M/10 with respect to sodium carbonate and then titrating back to the desired pH with 0.1 N HCl. The course of the hydrolysis was followed by a slight modification of the formol titration already described.<sup>6</sup> No marked effect of the pH could be noted. Several other experiments were

TABLE V.

*Effect of the Hydrogen Ion Concentration on the Inactivation of Trypsin by Inhibitor Solution.*

A. Gelatin, 5 per cent, containing 0.2 N  $\text{Na}_2\text{CO}_3$ . B. Gelatin, 5 per cent, containing 0.2 N  $\text{Na}_2\text{CO}_3$  and 4 per cent inhibitor solution. 50 cc. samples titrated to pH noted, and noted cc. trypsin added. Samples placed at 33°C. and formal titration run on 5 cc. at intervals.

pH at beginning.....	6.2	6.2	8.0	8.3	8.8	8.8	10.2	10.1
pH at end.....	6.2	6.2	7.5	7.6	7.6	8.0	9.8	9.7
Cc. inhibitor per 50 cc.....	0	2	0	2	0	2	0	2
Time in hours to cause increase of 1.0 cc. in formol titration.....	0.80	1.05	0.60	0.90	0.55	0.70	0.80	1.00
Per cent retardation.....	20		30		20		20	

made all of which gave results approximately the same as those given. No differences in the degree of retardation due to the pH were noted that could be definitely put outside the experimental error. This result was corroborated by the effect of the inhibiting solution on the rate of destruction of trypsin.<sup>2</sup> Here also no differences in the effect between pH 6 and 10 could be noted. This result seems to show that the "active" concentration of the trypsin and of the inhibiting substance is not markedly effected by the pH (between 6 and 10).

<sup>6</sup> Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 595.

*Does Trypsin Form a Compound with Gelatin?*

The hypothesis which has been found to account quantitatively for the experiments described in this paper contains the assumption that the velocity of hydrolysis is proportional to the concentration of *free* enzyme. That is, the enzyme would enter into the formula for the kinetics of the reaction just as would the hydrogen ion concentration in the case of a reaction catalyzed by hydrogen ions or as one of the reactants in any chemical reaction and the time presumably consists of the time required for a molecule of enzyme to come in contact with a molecule of gelatin. The idea was proposed by Brown<sup>7</sup> and has since been elaborated by various authors that enzymes combine with the substances which are hydrolyzed by them and that the velocity of the reaction depends on the speed of decomposition of this compound, while the time for the compound to form is negligible. If this were true in the case of trypsin it is clear that the mechanism for the equilibrium between trypsin and the inhibiting substance which has been found to agree with the experiments could not be verified since it would be necessary to allow for the amount of trypsin combined with the gelatin. If this mechanism is correct therefore it seems necessary to conclude that the amount of trypsin combined with the gelatin at any one time is negligibly small and that the limiting time element is really the time required for the compound to form as is the case in other chemical reactions. The same reasoning evidently applies to pepsin hydrolysis and to invertase (Euler<sup>4</sup>), since in both these cases it has been found that the equilibrium between the enzyme and an inhibiting substance can be quantitatively accounted for by the assumption that the rate of hydrolysis is proportional to the *free* enzyme. This question will be discussed in another paper.

*Can the Results be Accounted for by the Adsorption Formula?*

The adsorption formula as given by Freundlich<sup>8</sup> and as usually used is written

<sup>7</sup> Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

<sup>8</sup> Freundlich, H., *Kapillarchemie: Eine Darstellung der Chemie der Kolloide und verwandter Gebiete*, Leipsic, 1909.



$$\frac{x}{C \frac{1}{n}} = k \cdot m$$

where  $x$  is the amount adsorbed,  $C$  is the concentration of the substance remaining in the solution, and  $m$  is the amount of adsorbent (or the area of the adsorbing surface).  $k$  and  $n$  are constants. In the present experiments it cannot well be assumed that the enzyme is adsorbed by the inhibiting substance since there is no evidence that this is in other than true solution. It would be necessary to suppose therefore that the inhibiting substance is adsorbed by the enzyme. The amount of enzyme would therefore be represented by  $m$  in the above formula and there would be no way to determine how much was combined and how much was free since there is no term in the equation to represent the amount of the adsorbent (in this case the enzyme) which is combined.

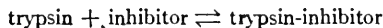
Hedin<sup>9</sup> has found that trypsin is adsorbed by charcoal and that the equilibrium in this case is that demanded by the adsorption formula. Hedin's experiments are, however, not contradictory to those described in this paper since it is quite possible that trypsin may be adsorbed by charcoal and yet react with other substances according to the law of mass action. This is known to be the case with acetic acid and many other substances.

#### SUMMARY.

1. A study has been made of the equilibrium existing between trypsin and the substances formed in the digestion of proteins which inhibit its action.

2. This substance could not be obtained by the hydrolysis of the proteins by acid or alkali. It is dialyzable.

3. The equilibrium between this substance (inhibitor) and trypsin is found to agree with the equation,



The equilibrium is reached instantaneously and is independent of the substrate concentration. If it be further assumed that the rate of

<sup>9</sup> Hedin, S., *Biochem. J.*, 1906, i, 484; *Z. physiol. Chem.*, 1906-07, i, 497.

hydrolysis is proportional to the concentration of the free trypsin and that the equilibrium conforms to the law of mass action, it is possible to calculate the experimental results by the application of the law of mass action.

4. The equilibrium has been studied by varying (*a*) the concentration of the inhibiting substance, (*b*) the concentration of trypsin, (*c*) the concentration of gelatin, and (*d*) the concentration of trypsin and inhibitor (the relative concentration of the two remaining the same). In all cases the results agree quantitatively with those predicted by the law of mass action.

5. It was found that the percentage retarding effect of the inhibiting substance on the rate of hydrolysis is independent of the hydrogen ion concentration between pH 6.3 and 10.0.

6. The fact that the experimental results agree with the mechanism outlined under 3, is contrary to the assumption that any appreciable amount of trypsin is combined with the gelatin at any one time; *i.e.*, the velocity of the hydrolysis must depend on the time required for such a compound to form rather than for it to decompose.

7. The experiments may be considered as experimental proof of the validity of Arrhenius' explanation of Schütz's rule as applied to trypsin digestion.

8. Inactivated trypsin does not enter into the equilibrium.

Many of the experiments described in this paper were carried out by Mr. Frank Johnston.

## THE INACTIVATION OF TRYPSIN.

### III. SPONTANEOUS INACTIVATION.

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In addition to the inactivation of trypsin caused by its combination with some of the products of the hydrolysis, which has been discussed in the first part of this series, trypsin undergoes a second or spontaneous inactivation. This inactivation is independent of the action of the enzyme, irreversible and distinct from the reversible retardation of the action of the enzyme by the products formed during the reaction. Tammann<sup>1</sup> clearly recognized this complicating factor and attempted to correct for it, and there has since been considerable discussion as to the nature and course of this reaction. The rate of destruction of a number of enzymes has been studied, especially by Madsen and Walbum<sup>2</sup> who found in general that the reaction was monomolecular.

The inactivation of trypsin was studied by Vernon<sup>3</sup> who found that the reaction was not monomolecular but became progressively slower than the rate predicted by the monomolecular formula. He concluded therefore that the solution must contain a number of different forms of the enzyme some of which were more stable than others. He also found that, as is generally the case, the purity of the solution had a marked influence on the rate of decomposition. As will be seen from the experiments in this paper it is this factor which causes the divergence from the monomolecular formula so that it is unnecessary to assume the existence of a series of enzymes differing in their degree of stability.

<sup>1</sup> Tammann, G., *Z. physik. Chem.*, 1889, iii, 25.

<sup>2</sup> See Arrhenius, S., *Immunochemie*, Leipsic, 1907.

<sup>3</sup> Vernon, H. M., *J. Physiol.*, 1904, xxx, 330.

*Methods.*

The methods used in the present experiments were the same as those described above. The amount of active trypsin present was determined by measuring the time required for 1 cc. of the solution to cause a small amount of hydrolysis of a gelatin solution at 33° and a pH of 6.2 (in one experiment the pH was 10). The hydrolysis was followed either by the formol titration or the change in conductivity.

*Influence of the containing vessels.*—Since the formula for a monomolecular reaction is the same as that for diffusion it is necessary to know whether the enzyme is really being destroyed or simply diffu-

TABLE I.

*Influence of Containing Vessel on Decomposition.*

10 cc. dialyzed trypsin placed in vessel noted, and left at 20°C., pH 6.2. trypsin determined in 1 cc. after interval noted.

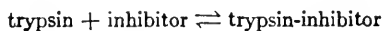
Time at 22°.	Trypsin per cc. after time noted at 22°C.			
	Containing vessel.			
	Platinum 5 cc.	Quartz 10 cc.	Paraffin 10 cc.	Glass 50 cc.
<i>hrs.</i>				
0	1.16	1.19	1.10	1.20
17	0.64	0.60	0.68	0.66

ing to the walls of the containing vessel. In the latter case the rate of disappearance of the enzyme will depend on the size and character of the containing vessel, while if the process is chemical it will probably be independent of these factors. In order to test this point trypsin solutions (prepared as described in the preceding paper) were placed in various vessels and kept at 22° for 17 hours. The activity of the solution was tested before and after this interval. The results of this experiment are shown in Table I. It is evident that the destruction of the enzyme is independent of the container and is therefore probably not a diffusion process. The same conclusion is indicated by the temperature coefficient.

*Influence of the Purity of the Solution on the Course of the Reaction.*

Fig. 1 contains the results of a series of experiments on the rate of inactivation of various trypsin solutions at 38° and pH 6.2. The amount of active trypsin remaining in solution at any time has been plotted as the logarithm so that if the reaction were monomolecular the resulting curve would be a straight line. As the figure shows this is true in the case of the dialyzed trypsin. This particular solution had been dialyzed under pressure for 18 hours at 6°C., filtered and redialysed. The constant found is 0.005 (time in minutes and common logs). This experiment could not be repeated with certainty but in general, the more carefully the solution was purified the more nearly the reaction was found to be monomolecular. The figure also shows that undialyzed trypsin solutions and those to which gelatin had been added are apparently inactivated at first more rapidly than the pure solutions and then much more slowly. On the other hand, solutions containing inactivated trypsin or substances which had been found to interfere with the action of the enzyme, are much more stable, and if a large amount of these substances are present amount of decomposition is too small to determine in the interval of time chosen. The addition of glycine is without effect.

It has been shown in the previous paper that the products formed by the action of trypsin on proteins form a compound with the trypsin that is inactive. The simplest explanation for the present experiments would be to assume that exactly the same mechanism is at work here and that the compound, trypsin-inhibitor, is stable as well as inactive. It was found that the experiments referred to were in quantitative agreement with the hypothesis that the trypsin and inhibiting substance unite to form a compound according to the equation



and further that this equilibrium conformed to the law of mass action; *i.e.*

$$\frac{\text{Concentration of free trypsin} \times \text{Concentration of free inhibitor}}{\text{Concentration of trypsin-inhibitor}} = \text{a constant}$$

It follows from this equation that the amount of free trypsin present in a solution containing a given amount of inhibitor is a function of the dilution. The more concentrated the solution the more trypsin will be combined. Since the determination of the amount of free trypsin was made by adding 1 cc. of the trypsin to 25 cc. of gelatin it is possible for most of the trypsin to be active (uncombined) under these conditions but nearly all combined (inactive and stable) in the undiluted trypsin solution.

If the trypsin solution at the beginning of the experiment contains undigested protein it is evident that the concentration of the inhibiting substance, which is found by the action of trypsin on the protein, will increase during the experiment, and that the observed result will be the combination of two effects: first, reversible inactivation of the trypsin due to the presence of the inhibiting substance, and second, irreversible destruction of the free trypsin. The combination of these two factors will give a curve which, compared to the curve for the pure trypsin solution, will drop too rapidly at first, due to the increasing concentration of inhibiting substance and will then decrease too slowly owing to the fact that the trypsin is nearly all combined and therefore stable. This is the result shown in Fig. 1. This explanation may be verified by diluting the solution sufficiently before determining its activity. Since the inactivation due to the inhibiting substance is reversible and depends on the concentration of the solution whereas the spontaneous inactivation is irreversible, the two effects may be separated in this way and the resulting value alone will be a measure of the irreversible spontaneous decompositions. The result of an experiment performed in this way is shown in Fig. 2. The curve for the formol titration of the trypsin solution alone is also given. The figure shows that when the trypsin solution is sufficiently diluted before the determination is made, the initial rapid drop disappears and also that the time during which this drop is noticeable in the less diluted solution corresponds to the time during which the formol titration is increasing.

There remains to be explained the subsequent retardation of the inactivation in solutions containing protective substances. It follows from the law of mass action which, as has been shown, correctly expresses the equilibrium, that the smaller the amount of trypsin

present the greater the percentage of combined and therefore stable trypsin. In a solution, therefore, originally containing trypsin and inhibiting (protective) substances, the percentage of the trypsin that

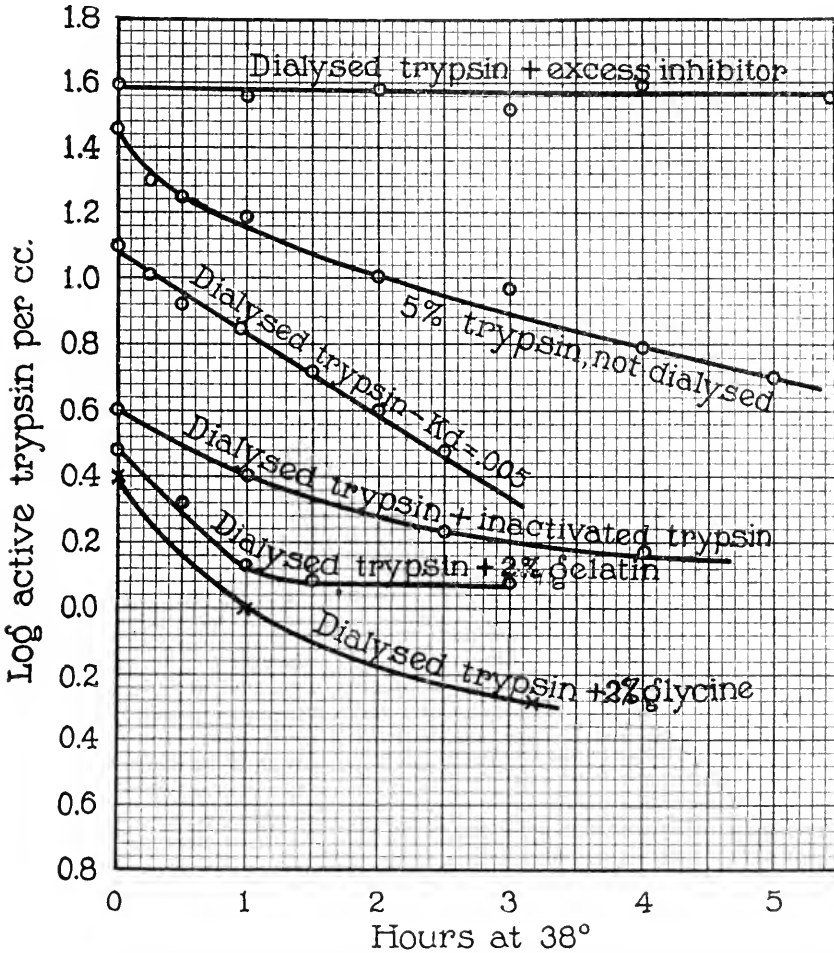


FIG. 1. Inactivation of various trypsin solutions at 38°C.

is free is constantly decreasing. Since it is this quantity that determines the rate of inactivation, the rate of inactivation will also constantly decrease and the resulting decomposition curve will fall more

slowly than demanded by the monomolecular formula. As has been stated this is the experimental result.

The above explanation may be tested quantitatively as follows. If the total concentration of trypsin and inhibitor be known the concentration of free trypsin at any dilution may be calculated by the law of mass action, since the equilibrium constant is known from the experiments described in the preceding paper. The constant

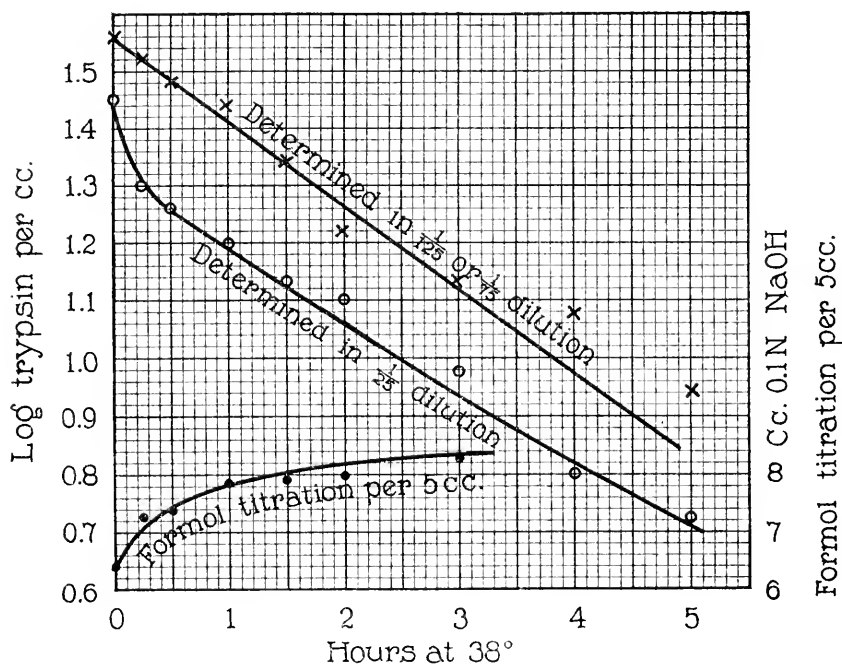


FIG. 2. Inactivation of trypsin in solution containing protein at 38°C.

for the destruction of the free trypsin is known from the decomposition curve of the purified trypsin. It was found to be about 0.005, using common logs and expressing the time in minutes. That is, very nearly half the amount of trypsin present is destroyed in 1 hour at 38° and a pH of 6.2. If a known amount of inhibitor is added to a known amount of trypsin, therefore, it is possible to calculate concentration of free trypsin in this solution. Since the value of  $K_d$  (the rate of decomposition) is known, the percentage of this free



trypsin that will be inactivated in any given interval of time can be approximated. The total amount of trypsin remaining in solution can then be found by difference and the amount of this which will be free and active at the concentration used in the determination, and in the presence of the known concentration of inhibitor can be calculated. This value should agree with that found by experiment. It must be remembered that the above calculation is only a first approximation since it contains two assumptions that are not strictly correct. (1) That the free trypsin is inactivated at the same rate as the same concentration of pure trypsin. As a matter of fact the amount of trypsin destroyed under the conditions of the experiment will be slightly greater than the quantity which would be destroyed if there were no combined stable trypsin present, since some of this will be dissociated as the free trypsin is destroyed and the amount of free trypsin and therefore the amount destroyed increased in this way. If the experiment is limited to the first part of the reaction (as was done) this difference is within the experimental error. (2) It was assumed that the only inhibiting (protective) substance present was added as the inhibiting solution. The decomposition curve of the trypsin solutions alone, however, show that in general there is always some protective substance present in the trypsin solution. The fact that the addition of inactivated trypsin renders the enzyme more stable indicates also that the solution contains some protective substances. This effect can hardly be ascribed to the inactive trypsin itself since it was shown in the first part of this paper that inactivated trypsin took no part in the reaction. The neglect of this quantity tends to make the calculated amount of trypsin destroyed too low. This is the result that is obtained. (If this quantity of inhibitor present in the trypsin solution is taken into account, the calculated and observed values may be made identical, but since there is no independent method of determining the value to be used, the process really consists in adding another arbitrary constant to the formula and so does not add much to the validity of the proof.)

The result of an experiment calculated and carried out as described above is shown in Table II. The agreement is as good as could be expected in view of the many sources of experimental error and of the fact that the calculation involves the extrapolation of the equilib-

rium equation over a range of 2,500 per cent dilution, since the values for the constants were determined in a solution which had been diluted 25 times and which in addition contained gelatin, whereas in the calculation the same formula was applied to a solution which had not been diluted at all and which contained no gelatin. This experiment appears to furnish strong confirmation of the validity

TABLE II.

*Concentration of Inhibitor and Rate of Inactivation of Trypsin at 38°C.*

10 cc. dialyzed trypsin and noted units inhibitor placed at 38°C. 1 cc. removed after intervals noted and added to 25 cc. of 2 per cent gelatin pH 6.2, specific conductivity  $2.2 \times 10^{-3}$ , at 33°C. and rate of hydrolysis followed; this gives the units of free trypsin per cc. solution when diluted 1:26.  $K = 0.1$ .  $Kd$  (decomposition trypsin) = 0.005.

Experiment No.	Inhibitor per cc. trypsin solution.	Free trypsin per cc. solution diluted 1:26 after hours at 38°C.					
		0 hours.		0.6 hours.		1.0 hours.	
		Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.
	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>
1	0	3.45	(3.45)	2.6	2.5		
1	0.17	3.40	3.35	3.0	2.3		
1	0.50	3.0	3.2	2.9	2.2		
1	1.7	2.3	2.6	2.2	2.15		
		2.5					
2	0	2.5	(2.5)			1.1	1.25
2	1.7	1.9	1.8			1.7	1.5
		2.0					
3	0	3.3	(3.3)			1.65	1.65
3	1.8	2.7	2.75			2.2	1.8

of the mechanism proposed for the reaction between the trypsin and inhibitor. It also shows that the equilibrium is not effected to any extent by the gelatin. The conclusion seems unavoidable that little or no trypsin is combined with the gelatin. The fact that gelatin has no protective influence on the trypsin also points to the same conclusion.

*The Influence of the Manner in which the Inhibiting Solution is Added.*

It was found in the study of the effect of the inhibiting solution on the rate of digestion by trypsin that the order in which the solutions were mixed and the time of standing was without effect on the result provided the control solution of pure trypsin did not alter during the experiment. If, however, the experiment is made at 38° where the control solution is rapidly destroyed the results are very different. The results of such an experiment are shown in Fig. 3. It will be seen that the solution to which all of the inhibitor had been added at the beginning of the experiment is much more active at the end than the one to which the inhibitor was added at intervals. This is evidently very similar to the Danysz phenomenon in immunology. In the present experiment the result is more marked if a relatively small quantity of the inhibitor ( $\approx$  antitoxin) is added at the beginning of the experiment whereas in the Danysz phenomenon it is necessary to add an excess. This is due to the fact that in the present experiment it is the free trypsin ( $\approx$  toxin) that is irreversibly changed during the experiment while in the Danysz experiment it is apparently the free antitoxin ( $\approx$  inhibitor) which is altered.<sup>2</sup>

*The Decomposition of the Trypsin-Inhibitor Compound.*

The rate of destruction of this compound at 38° is so slow in comparison to that of the free trypsin that it may be neglected. At 62°, however, it decomposes quite rapidly and follows the course of a monomolecular reaction (as was to be expected) provided an excess of inhibitor has been added so that practically all the trypsin is combined in the undiluted solution. The result of an experiment is given in Fig. 4. Irregular results were obtained in some cases owing to the formation of a precipitate presumably of coagulated protein. In such cases the amount of active trypsin remaining in solution shows a sudden drop at the time of formation of the precipitate.

*Influence of the pH on the Rate of Decomposition.*

Fig. 5 contains the result of an experiment in which the trypsin solutions were adjusted to various pH by the addition of HCl or NaOH before being placed at 38° for 1 hour. The activity of the

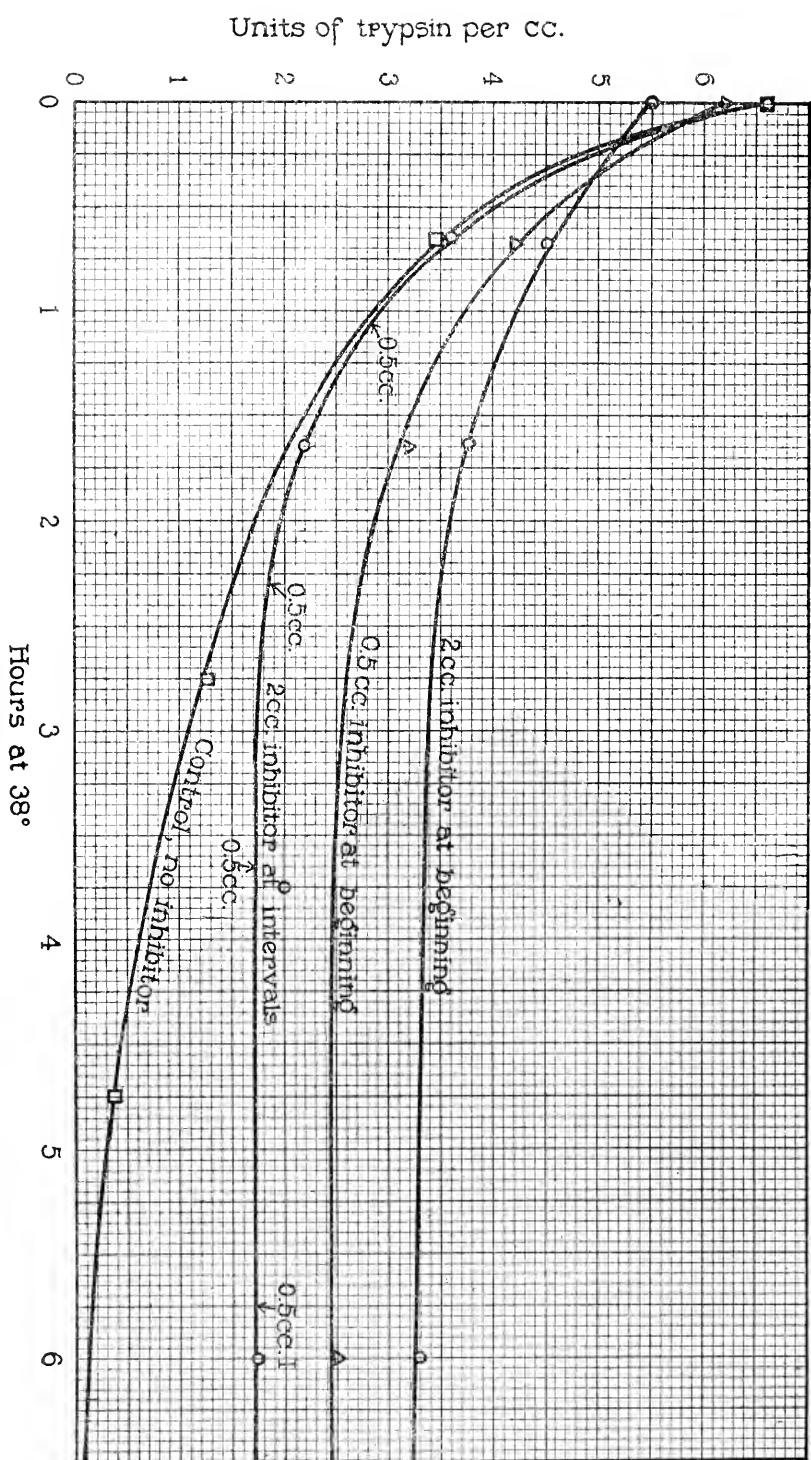


FIG. 3. Effect of time of adding inhibitor on decomposition of trypsin at 38°C.

solutions was determined by adding 1 cc. to 50 cc. of 5 per cent gelatin containing 0.2 M  $\text{Na}_2\text{CO}_3$  (pH = 10) and the rate of hydrolysis followed by the formol titration. Trypsin is evidently most stable at a pH of 5. The rate of decomposition increases quite rapidly if the solution is either more or less acid.<sup>4</sup> It differs in this respect from pepsin which is stable over quite a wide range and resembles the invertase studied by Hudson.<sup>5</sup> The rapid increase in the rate of destruction

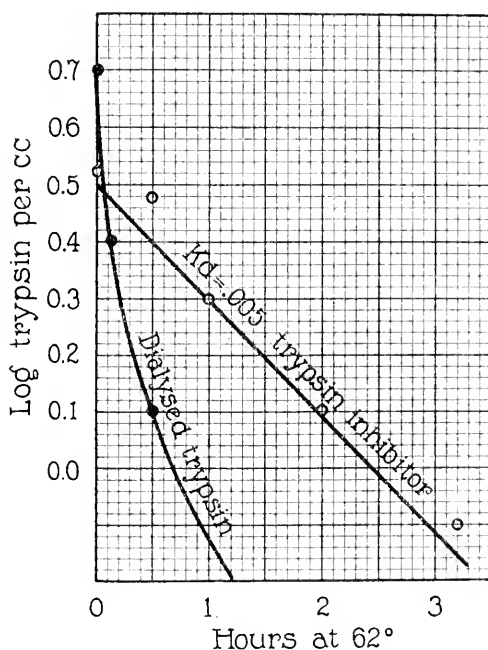


FIG. 4. Inactivation of trypsin and trypsin-inhibitor compound at 62°C.

with increasing alkalinity makes it evident that this behavior must enter to a large extent in deciding the optimum pH for the action of the enzyme. It was not found possible to determine quantitatively the effect of the pH owing to the fact that the reactions are rarely strictly monomolecular and it is therefore difficult to find a value

<sup>4</sup> This agrees with the experiments of Ringer (Ringer, W. E., *Z. Physiol. Chem.*, 1921, cvi, 107).

<sup>5</sup> Hudson, C. S., and Paine, H. S., *J. Am. Chem. Soc.*, 1910, xxxii, 774.

which correctly expresses the rate of decomposition. The time required for a certain amount to be destroyed could not be determined owing to experimental difficulties.

*Influence of the pH on the Protective Effect of the Inhibiting Substance.*

A comparison of the curves (Fig. 5) for the dialyzed trypsin solution and the solution to which inhibiting substance had been added shows that the protective action of the latter is also a function of the pH. There is little or no protective action on the acid side of pH 5.

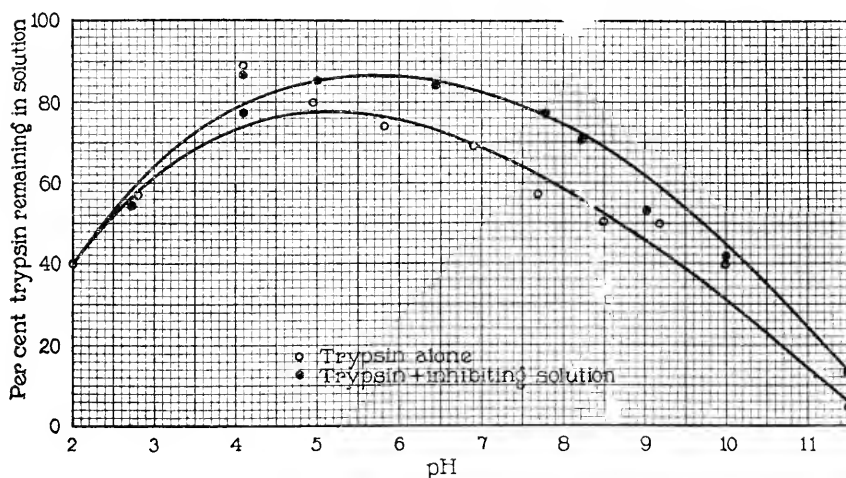


FIG. 5. Decomposition of trypsin solutions at 38°C. and different hydrogen ion concentrations. Per cent of trypsin remaining active after 0.5 hours at 38°C.

As the solution becomes more alkaline the protective action increases and then decreases slightly although the experiments are hardly accurate enough to be certain of this second decrease. If the hypothesis which has been used to account for experiments so far is correct this behavior evidently means that on the acid side of pH 5, trypsin does not combine with the inhibiting compound and that the combination has a maximum somewhere near a pH of 8 to 9.

This experiment confirms those described in the preceding paper in which it was found that the retarding influence of the inhibiting solu-

tion was not markedly effected by variations in the pH of the solution in the range of pH 6 to 10.

The mechanism which has been found to agree with the experiment described in this paper will also account for a peculiar fact which has been frequently observed in the study of the destruction of enzyme; namely, that the rate of decomposition at any one concentration will be strictly monomolecular, but that the rate becomes increasingly greater the more dilute the solution, instead of being independent of the concentration as is demanded by the monomolecular formula. If, as has been shown to be true for trypsin, the rate of decomposition depends on the amount of uncombined enzyme, it follows that the more dilute the solution the more rapidly the enzyme will become inactivated since the enzyme-inhibitor compound dissociates with increasing dilution. If, further, the inactivated enzyme reacts with the inhibitor to the same extent as does active enzyme (which was found to be the case with pepsin) then the rate of decomposition will be strictly monomolecular at any one concentration but will be the greater the more dilute the solution. This is the experimental result.

#### SUMMARY.

1. The rate of inactivation of purified trypsin solutions approximates closely that demanded by the monomolecular formula. The more carefully the solution is purified the closer the agreement with the formula.

2. The products formed by the action of trypsin on proteins renders the trypsin more stable. Gelatin and glycine have no effect.

3. The rate of inactivation of trypsin solutions containing these products does not follow the course of a monomolecular reaction but becomes progressively slower than the predicted rate.

4. The protective action of these substances is much greater if they are added all at once at the beginning of the experiment than if they are added at intervals.

These observations may be quantitatively accounted for by the hypothesis that a compound is formed between trypsin and the inhibiting substance which is stable as well as inactive, and that the rate of decomposition depends on the amount of uncombined trypsin present.

5. Trypsin is most stable at a pH of 5 and is rapidly destroyed in strongly acid or alkaline solution.

6. The protective effect of the inhibiting substances is small on the acid side of pH 5, increases from pH 5 to 7, and then remains approximately constant.



## DIRECT AND INDIRECT DETERMINATIONS OF PERMEABILITY.

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(Received for publication, November 21, 1921.)

Lack of direct and satisfactory means of determining the penetration of substances into the living cell has greatly hampered the study of permeability. Various indirect methods have been resorted to, but their reliability must remain in question until a direct method can be found for testing them.

Investigators have sought to overcome this difficulty in various ways. Some have made analyses of tissues, but it is evident that these must include too much intercellular material to be satisfactory. Analysis of the solution in which the tissue lies, in order to determine what is absorbed, is open to the objection that substances collect on the surfaces of cells, as well as in the cell walls and in the spaces between them, so that it is impossible to say what actually penetrates the protoplasm. Others have sought to analyze the cell sap. Plant cells are most favorable for this purpose, since, as a rule, they contain vacuoles filled with sap. In general the method has been to crush the tissues and express the sap, but this procedure involves many possibilities of error.<sup>1</sup>

<sup>1</sup> Among these may be mentioned contamination of the cell sap by substances present in the cell walls or intercellular spaces and chemical reaction between the cell sap and the crushed protoplasm or the cell walls. The degree of pressure used in expressing has a marked influence on the concentration of the sap (cf., Mameli, E., *Atti del r. inst. bot. de Pavia*, 1908, xii, 285; Dixon, H. H. and Atkins, W. R. G., *Sci. proc. roy. Dublin Soc. N. S.* 1913, xiii, 422; Gortner, R. A., Lawrence, J. V., and Harris, J. A., *Biochem. Bull.*, 1916, v, 139).

The investigation of blood and other body fluids is open to the objection that we do not know to what extent substances penetrate between the cells in reaching these fluids. In many cases penetration into these fluids seems to present very special features.

The taking up of dyes has been extensively investigated but this method is beset by many pitfalls<sup>2</sup> and the results hitherto obtained are confusing.

In some cases the penetration of acids and alkalies has been studied by means of organisms containing natural indicators, or by introducing indicators into the cell.<sup>3</sup> Use has also been made of the fact that the penetrating substance may cause a visible precipitate within the cell; this is especially the case with alkaloids.<sup>4</sup> Furthermore the absorption of  $\text{Ca}^{5+}$  has been detected by observing the formation of crystals of  $\text{Ca}$  oxalate within the cell. It is evident, however, that these methods have but limited application, and that in many cases they are open to the objection that the penetrating substance injures the cell.

The penetration of a substance may sometimes be demonstrated by observing its effect upon metabolism, but this method is inadequate from a quantitative standpoint. Some investigators contend that substances may produce effects on metabolism by their action at the surface, without actually penetrating the cell.

<sup>2</sup> To a great extent the coloration of the cell by a dye shows the extent to which the dye can combine with the substances within the cell rather than the rate at which the dye penetrates. Thus many cells contain substances which combine with methylene blue so that it becomes far more concentrated within the cell than in the external solution (Pfeffer, W., *Physiology of plants*, 2nd edition, Oxford, 1897, i, 96). Unless the cell has this power it often fails to appear colored even though it may contain the dye in the same concentration in which it exists outside. In such cases it may sometimes be detected by plasmolyzing the cell and thus concentrating the dye. A further complication is that a cell may appear to have taken the dye into its interior when in reality only the surface or the cell wall is stained. There are many other difficulties, which need not be discussed here, such as toxic action of the dye and changes in the dye (decolorization, etc.) as it enters the cell. A very serious objection to this method is that it does not give quantitative results. A review of the literature will be found in Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, 4th edition, Leipsic and Berlin, 1914.

<sup>3</sup> For the literature see Haas, A. R., *J. Biol. Chem.*, 1916, xxvii, 233; Crozier, W. J., *J. Biol. Chem.*, 1916, xxiv, 255; xxvi, 225; 1918, xxxiii, 463.

<sup>4</sup> For the literature see Czapek, F., *Ueber eine Methode zur direkten Bestimmung der Oberflächenspannung der Plasmahaut von Pflanzenzellen*, Jena, 1911.

<sup>5</sup> Osterhout, W. J. V., *Z. physik. Chem.*, 1910, lxx, 408.

It is evident that the most satisfactory method is to place the cell in a solution containing the substance whose penetration is to be investigated and, after a definite time of exposure, to obtain the cell sap without contamination and test it for the presence of this substance. Experiments of this sort have apparently not been carried out, though interesting results have been obtained by Meyer,<sup>6</sup> Hansen,<sup>7</sup> Wodehouse,<sup>8</sup> and Crozier<sup>9</sup> by comparing the cell sap of the marine alga *Valonia* (which can be obtained without contamination) with the sea water. Janse<sup>10</sup> found that filaments of *Spirogyra* which had been kept for a time in a solution of  $\text{KNO}_3$  gave a test for  $\text{NO}_3$  after being rinsed and caused to burst in a solution of diphenylamine. In this method there is serious risk of contamination by substances in and upon the cell wall (or between the cell wall and the protoplasm).

In order to avoid this difficulty the writer has employed the large, multinucleate cells of a species of *Nitella*, some of which reach a length of 6 inches and a diameter of a thirty-second of an inch.

Within the cell wall the protoplasm forms a thin layer in which the chlorophyll bodies are imbedded. Inside this layer is the large central vacuole filled with cell sap. It is possible to obtain the cell sap without contamination in various ways. The writer has made use of the following methods: The cells are placed for the desired length of time in a solution containing the substance whose penetration is to be tested. They are removed, washed in running tap water (followed in many cases by distilled water), and dried by means of filter paper. The cells are so large and turgid that this manipulation presents no difficulty. A cell is then placed on a piece of glass or filter paper and pierced with the point of a clean capillary tube (which has been drawn out to a fine tip). The cell sap is drawn up into the tube (by capillary action) quite free from protoplasm or chloroplasts.<sup>11</sup> Another method, which is preferable in many cases,

<sup>6</sup> Meyer, A., *Ber. deutsch. bot. Ges.*, 1891, ix, 77.

<sup>7</sup> Hansen, A., *Mitt. Zool. Stat. Neapel*, 1893, xi, 255.

<sup>8</sup> Wodehouse, R. P., *J. Biol. Chem.*, 1917, xxix, 453.

<sup>9</sup> Crozier, W. J., *J. Gen. Physiol.*, 1918-19, i, 581.

<sup>10</sup> Janse, J. M., *Versl. Meded. Kon. Akad. Wetensch. Afd. Natuurk.* (3), 1888, iv, 332.

<sup>11</sup> During the manipulation care should be taken to prevent the sap from running out of the cell and coming in contact with its outer surface.

is to suspend the cell by a pair of forceps attached to the upper end, cut off the lower end and bring it in contact with a glass slide, and then grasp the upper end gently with another pair of forceps which is slowly moved downwards while a slight pressure is maintained. The cell sap then flows out on to the glass slide. By uniting the drops from a number of cells it is possible to get a sufficient amount for qualitative chemical tests, and in many cases approximate quantitative results may be obtained.

Since in previous investigations the writer had employed indirect methods of testing permeability it was of considerable interest to compare such results with those obtained by direct tests of the cell sap. An investigation was therefore made in which the permeability of *Nitella* was tested by determinations of plasmolysis and of electrical conductivity as well as by the direct method. This may be illustrated by a series of experiments<sup>12</sup> with  $\text{NaNO}_3$  and  $\text{Ca}(\text{NO}_3)_2$ .

Experiments on plasmolysis were carried out by placing the cells in a hypertonic solution and observing the time required to recover from plasmolysis (without removing the cells from the solution) on the assumption that the more rapid the recovery the more rapid is the penetration of the salt.

In these experiments the smaller cells near the tip of the plant were largely employed. They were observed in Syracuse watch-glasses, or placed on glass slides and covered with large cover-glasses the edges of which were sealed with vaseline. The experiments require continuous observation of individual cells, since (especially in unbalanced solutions) recovery is promptly followed in many cases by injury and false plasmolysis,<sup>13</sup> which may be mistaken for true plasmolysis.

It is evident that plasmolysis may be injurious to many cells even in a balanced solution;<sup>14</sup> while in an unbalanced solution there may be

<sup>12</sup> All the experiments were performed at about 19°C. All the solutions were approximately neutral.

<sup>13</sup> Osterhout, W. J. V., *Bot. Gaz.*, 1908, xlv, 53; 1913, lv, 446; *Science*, 1911, xxxiv, 187.

<sup>14</sup> For this reason penetration may be more rapid than would otherwise be the case. In order to reduce toxicity pure salts should be used and the water should be distilled from quartz (or from glass which has been in use for some time), using cotton plugs in place of rubber or cork stoppers, and rejecting the first and last parts of the distillate.

the additional injury due to the toxic action of the salt. For this reason many cells which would recover if very slightly plasmolyzed may not do so if plasmolyzed more strongly, since recovery may require so much time that the process of injury gets the upper hand.

It was found that recovery was more rapid in  $\text{NaNO}_3$  than in a balanced solution of  $\text{NaNO}_3$  plus  $\text{Ca}(\text{NO}_3)_2$  or in  $\text{Ca}(\text{NO}_3)_2$  or  $\text{CaCl}_2$  alone. Experiments with  $\text{RbCl}$  and  $\text{CsCl}$ , with and without the addition of  $\text{CaCl}_2$ , gave similar results. This indicates that in a solution of  $\text{NaNO}_3$ ,  $\text{NaCl}$ ,  $\text{RbCl}$ , or  $\text{CsCl}$  penetration is more rapid than in  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{CaCl}_2$ , or a balanced solution.

These results agree with those previously obtained by the writer in studying *Spirogyra*.<sup>15</sup>

The experiments on conductivity were carried out by means of an apparatus consisting of a block of paraffin (*P*, Fig. 1) in which are two depressions containing platinum electrodes, *E*, *E*, covered with platinum black. A connecting groove contains the cells, *N*, loosely packed together and covered by a glass plate, *G*. The cells are placed in the groove after it is filled with liquid and great care is taken to avoid air bubbles. When a new solution is to be introduced it is poured into *T*; it then runs through the groove and escapes by the opening *O*; the irrigation is continued until the old solution is completely removed.

Before beginning the experiments the conductivity of the cell sap was tested<sup>16</sup> and found to be equivalent to that of sea water diluted with three parts of tap water (this will be referred to as 0.25 sea water). As the cells grew well in 0.20 sea water they were kept in this for some time before each experiment and then transferred to 0.25 sea water just before the experiment. The conductivity of the cell sap was then approximately the same as that of the external solution and the fact that the cells had a greater resistance than the solution must be ascribed to the greater resistance of the protoplasm or of the cellulose wall. When the cells were killed by agents which produce no alteration in the cell wall the resistance fell to approximately that of the solution. It is therefore evident that the increased resistance was due to the protoplasm.

<sup>15</sup> Osterhout, W. J. V., *Science*, 1911, xxxiv, 187.

<sup>16</sup> For this purpose a capillary tube was filled with sap and a platinum electrode was inserted into each end of the tube, care being taken to avoid air bubbles.

The conductivity was first determined in 0.25 sea water, this was then replaced by another solution of the same conductivity. The conductivity in the new solution was then determined at intervals.

In general it was found that in solutions in which the cells lived well, such as dilute sea water or a balanced solution of  $\text{NaNO}_3$  plus  $\text{Ca}(\text{NO}_3)_2$ , the resistance did not fall. But in solutions in which injury occurred, such as  $\text{NaNO}_3$ , the resistance fell. In  $\text{Ca}(\text{NO}_3)_2$  and in  $\text{CaCl}_2$  there was a rise in resistance followed by a fall.

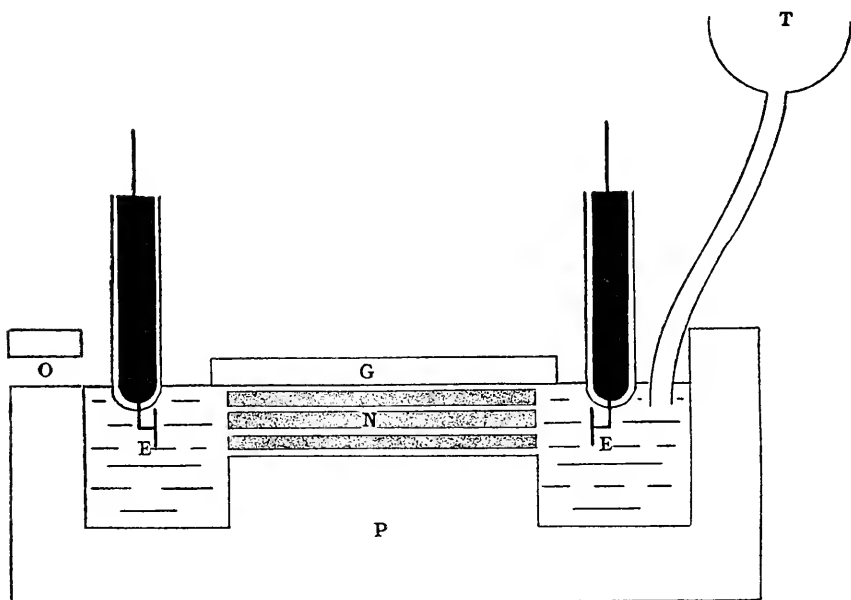


FIG. 1. Apparatus for measuring the conductivity of *Nitella*. The cells (*N*) are placed in a trough in a block of paraffin (*P*) and covered with a glass plate (*G*). The solution is poured in through the funnel (*T*) and runs out through the opening (*O*). At *E* and *E* are platinum electrodes.

These experiments agree with those made on *Laminaria* except that the rise observed in  $\text{Ca}(\text{NO}_3)_2$  or in  $\text{CaCl}_2$  was often smaller in the case of *Nitella*. This, however, is not unexpected, since the concentration of  $\text{CaCl}_2$  was only one-fourth of that used in the experiments on *Laminaria*. It may be added that the rise observed in  $\text{CaCl}_2$  differs greatly in different organisms. In *Rhodymenia*,<sup>17</sup>

<sup>17</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1918-19, i, 299.

for example, it is very small and temporary, even in 0.278 M  $\text{CaCl}_2$  (which has the conductivity of sea water).

As was to be expected from experiments on other organisms<sup>18</sup>  $\text{La}(\text{NO}_3)_3$  also produces a rise in resistance which is followed by a fall.

One fundamental principle is evident in the results of all the experiments, including those on plasmolysis and on electrical conductivity; namely, that in solutions which are injurious the resistance eventually falls, while in non-toxic solutions it remains practically unaltered. This has been interpreted as indicating that injury is accompanied by an increase of permeability, but the evidence for this view was obtained by indirect methods and the writer welcomed the opportunity to test it by the direct method of examining the cell sap.

The experiments on sap were confined to the determination of  $\text{NO}_3$ , since it was found that the cells normally give tests for Na and Ca. Since the method employed was not sensitive enough to detect  $\text{NO}_3$  in the sap of the control cells under any circumstances, it is evident that if a test was obtained after exposure to a solution containing  $\text{NO}_3$  it must have been due to penetration from without.

The sap was tested by placing it on a glass slide, adding a drop of nitron dissolved in 10 per cent acetic acid, and observing it under the microscope. If  $\text{NO}_3$  is present it may be recognized by the formation of characteristic crystals.

Cells kept for 24 hours in 100 cc.  $\text{NaNO}_3$  0.05 M plus 10 cc.  $\text{Ca}(\text{NO}_3)_2$  0.05 M gave no test, which shows conclusively that the method is safe as far as contamination by  $\text{NO}_3$  on the surface is concerned. After 48 hours a test was obtained. As the cells continued to live in this solution for 3 weeks (at which time the experiment was discontinued) and as they appeared normal in every way, it is evident that the penetration was not the result of injury.

It is probable that in 24 hours there was some penetration which was not revealed by the test. This, however, has no significance in the present investigation, the aim of which is not to determine the absolute amount of penetration but merely to compare the relative penetration in balanced and unbalanced solutions.

The results of such a comparison are very striking. After 3 hours in  $\text{NaNO}_3$  0.05 M a good test was obtained. The cells had lost some

<sup>18</sup> Osterhout, W. J. V., *Bot. Gaz.* 1915, lix, 464; *J. Gen. Physiol.* 1918-19, i, 299, 409.

of their turgidity; if left in the solution of  $\text{NaNO}_3$  or if transferred to tap water they subsequently lost all their turgidity, indicating death. It is therefore evident that this rapid penetration was accompanied by injury.

It may be remarked that the turgidity of the cells is a good indication of their condition. It is easily tested by lifting them out of the solution; if in good condition they appear stiff, if injured they tend to collapse. It is, however, necessary to distinguish between loss of turgidity in isotonic or hypotonic solutions, which indicates injury, and a similar appearance in hypertonic solutions, which may indicate nothing of the sort. In the latter case the cell promptly recovers its turgidity when placed in tap water; in the former it does not.

Another criterion of injury is afforded by the appearance of the chlorophyll bodies. In the normal cell they are arranged in regular rows and are of a clear, transparent green color. When injury occurs they lose their regular arrangement and their color becomes more opaque.

In 0.05 M  $\text{Ca}(\text{NO}_3)_2$  the cells live for a week or more. During the first few days, at least, penetration is not more rapid (perhaps is less so) than in a balanced solution.

Similar results were obtained with other salts, which will be described in a subsequent paper.

The outcome of these direct tests is therefore a confirmation of the results obtained by the indirect methods. We find that penetration in injurious solutions is relatively rapid as compared with penetration in non-toxic solutions. This corresponds to the fact that recovery from plasmolysis is more rapid in injurious solutions as well as to the fact that conductivity increases in such solutions.

In view of this we may conclude that determinations of electrical conductivity give reliable information regarding changes in permeability. Observations on recovery from plasmolysis, while giving similar results, are less satisfactory.

#### SUMMARY.

1. Methods are described for obtaining cell sap from *Nitella* without contamination.



2. Tests of the cell sap show that in a balanced solution of  $\text{NaNO}_3$  plus  $\text{Ca}(\text{NO}_3)_2$  there is a slow penetration of  $\text{NO}_3$  and that the cell remains in a normal condition, but in pure  $\text{NaNO}_3$  there is rapid penetration accompanied by injury.

3. Inasmuch as determinations of electrical conductivity give the same result it may be concluded that this method gives reliable information regarding permeability.

4. While observations on recovery from plasmolysis give similar results, the method is less satisfactory.



## STUDIES ON BIOLUMINESCENCE.

### XIV. THE SPECIFICITY OF LUCIFERIN AND LUCIFERASE.

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(Received for publication, October 15, 1921.)

#### INTRODUCTION.

In the living world are found more than thirty groups of organisms producing light, scattered, in the animal kingdom at least, through orders widely different in morphological characteristics. Physiologically, one order may contain individuals producing light in quite different ways, as the squids, some of which have light organs producing an external secretion of luminous material, while others possess light organs of internal combustion. Among the fish, also, are found various types of luminescence. Some forms emit light only as a result of stimulation while others luminesce continually day and night, and the intensity is quite independent of stimulation of any kind. In this respect these fish resemble the fungi and luminous bacteria which also emit a steady continuous light which is not varied on stimulation.

While it is impossible at the present time, because of lack of data, to classify accurately and logically the various types of luminescence found in living things, the accompanying list is an attempt in this direction, which will serve as a guide to the groups, whose particular characteristics of luminescence are discussed in this paper. Accordingly, relationship as well as physiological peculiarities of luminescence have been considered in making the group (Table I).

Two questions at once arise in connection with these data. The first has to do with the presence of the luciferin-luciferase reaction. As is evident from the above list, luciferin and luciferase, first dis-

TABLE I.  
*Groups of Luminous Organisms.*

Group.	Genus studied.	Type of luminescence.	Luciferin and luciferase.
Bacteria.	{ <i>Microspira.</i> <i>Photobacterium.</i> }	Intracellular and continuous.	Negative.
Fungi.	{ <i>Armillaria.</i> <i>Clytocybe.</i> }	Intracellular and continuous.	?
Radiolaria.	<i>Thalassicola.</i>	Intracellular; on stimulation.	?
Dinoflagellates.	{ <i>Gonyaulax.</i> <i>Ceratium.</i> }	Intracellular; on stimulation.	?
Cystoflagellates.	<i>Noctiluca.</i>	Intracellular; on stimulation.	Negative.
Sponges.	<i>Grantia.*</i>	(?); on stimulation.	Negative.
Medusæ and hydroids.	{ <i>Æquorea.</i> <i>Mitrocoma.</i> }	Extracellular (?); on stimulation.	Negative.
Pennatulids.	{ <i>Cavernularia.</i> <i>Pennatula.</i> <i>Ptylosarcus.</i> }	Extracellular (?); on stimulation.	Negative.
Ctenophores.	<i>Bolina.</i>	Intracellular (?); on stimulation.	Negative.
Tomopterid worms.	<i>Tomopteris.</i>	Extracellular (?); on stimulation.	?
Syllid worms.	<i>Odontosyllis.</i>	Extracellular (?); on stimulation.	Positive.
Polynœd worms.	<i>Polynœ.</i>	Intracellular; on stimulation.	Negative ?
Chætopteroid worms.	<i>Chætopterus.</i>	Extracellular; on stimulation.	Negative
Earthworms.	<i>Microscolex.</i>	(?); on stimulation.	?
Ostracod crustacea.	{ <i>Cypridina.</i> <i>Pyrocypis.</i> }	Extracellular; on stimulation.	Positive.
Copepod crustacea.	{ <i>Metridia.</i> <i>Pleuromma.</i> }	Extracellular; on stimulation.	?
Schizopod crustacea.	{ <i>Meganyctiphanes.</i> <i>Gnathophausia.</i> }	Intracellular; on stimulation. Extracellular; on stimulation.	Negative. ?

\* Luminescence doubtful.

TABLE I—*Continued.*

Group.	Genus studied.	Type of luminescence.	Luciferin and luciferase.
Decapod crustacea.	<i>Sergestes.</i>	Intracellular; on stimulation.	?
	<i>Heterocarpus.</i>	Extracellular; on stimulation.	?
Myriapods.	<i>Geophilus.</i>	Extracellular; on stimulation	Negative?
Springtails.	<i>Podura.</i>	Intracellular (?); on stimulation.	?
Beetles.	<i>Luciola.</i> <i>Pyrophorus.</i>	Intracellular; on stimulation.	Positive
Flies.	<i>Boleophila.</i>	Intracellular; on stimulation.	?
Brittle stars.	<i>Ophiurantha.</i>	Intracellular (?); on stimulation.	?
Lamellibranch mollusks.	<i>Pholas.</i>	Extracellular; on stimulation.	Positive.
Nudibranch mollusks.	<i>Phyllirrhoe.</i>	Intracellular (?); on stimulation.	?
Cephalopods.	<i>Watasenia.</i>	Intracellular; on stimulation.	Negative.
	<i>Heteroteuthis.</i>	Extracellular; on stimulation.	?
Balanoglossids.	<i>Glossobalanus.</i>	Extracellular; on stimulation.	Positive.
Salpids.	<i>Pyrosoma.</i>	Intracellular; on stimulation.	Negative.
Fish	<i>Photoblepharon.</i> <i>Anomalops.</i>	Intracellular; continuous.	Negative.
	<i>Maurolicus.</i> <i>Porichthys.</i>	Intracellular; on stimulation.	?
	<i>Gonostoma.</i>	Extracellular (?); on stimulation (?).	?

covered by Dubois<sup>1</sup> in *Pyrophorus*, an elaterid beetle, and *Pholas*, a mollusk can be demonstrated in several other groups of the animal kingdom but not in all. Why cannot these substances be demonstrated in all orders? It would seem that so fundamental a reaction should be universal; that is one question awaiting solution.

A second question concerns the specificity of luciferin and luciferase. Will the luciferase of one species produce light with the luciferin of another species, or genus, or group, and *vice versa*? We have here material for an interesting study of enzyme specificity and this is necessary, as we shall see, for a proper analysis of the first question, why luciferin and luciferase cannot be demonstrated in all groups of luminous animals.

#### *The Luciferin-Luciferase Reaction.*

The general methods for preparing luciferin and luciferase are very simple. Luciferin is made by adding hot water to the luminous organ of the animal or by quickly heating the luminescent extract of the luminous animal to temperatures which permanently quench the light, or to boiling. By this means the luciferase is destroyed on heating before the luciferin (which is not destroyed by heating) has been completely oxidized. Care must be taken to destroy the luciferase as quickly as possible, before it has had time to oxidize the luciferin. Hence the advantage of adding hot water suddenly to the luminous gland. Care must also be taken not to heat the luciferin to too high a temperature, or too long, as it may be destroyed under these conditions. Hence the advantage of heating a luminous extract to just the point where the light is permanently extinguished, and cooling quickly. Before deciding that luciferin cannot be demonstrated in an animal, these precautions have always been taken.

Luciferase is prepared by allowing a cold water extract of the luminous gland to stand until the luciferin has been completely oxidized. This oxidation can be accelerated by shaking the solution to aerate it well, or by gentle heating (not sufficient to destroy the luciferase), or by adding such substances as chloroform, saponin, or sodium glycocholate. These substances apparently act by liberating

<sup>1</sup> Dubois, R., *Compt. Rend. Soc. Biol.*, 1885, ii, 559; 1887, iv, 564.

luciferin bound (combined or absorbed) in some way in the solution, perhaps sometimes by causing cytolysis of still intact photogenic cells or by causing solution of photogenic granules or granulysis. Extracts of non-luminous animals sometimes contain substances acting like the above cytolytic agents. For these I have suggested the general term of photopheleins. Care has been taken to exclude such sources of error and misinterpretations in the studies described below.

It is obvious, from the method of preparation of luciferase, that, should there be just enough luciferase or less than enough luciferase to oxidize all the luciferin of a luminous gland, we could not obtain a solution of luciferase by the above method. Only if an excess of luciferase over luciferin exists can a solution of luciferase be obtained. It is possible, therefore, that this is the explanation of negative results for the presence of these bodies in certain groups of luminous organisms, a possibility that can be tested in part and that will be discussed below.

It is not to be supposed that inability to demonstrate luciferin and luciferase in a luminous form is always due to the same cause. Assuming that luciferin and luciferase really do occur in all luminous forms, it may be that they are present in such small amounts, compared with the bulk of non-luminous tissue necessarily included in extracting them, that no luminescence is visible. This might be the case in *Chaetopterus*, an annelid worm, where luminous gland cells occur over the surface of the body. These cells cannot be removed individually and the most luminous regions of the worm must be extracted as a whole, involving a large mass of non-luminous material.

Again, either luciferin or luciferase or both may be very unstable in some forms, undergoing change before their presence in an extract may be tested.

Or, it is not impossible that the luciferase may occur in an endoenzyme condition, similar to the zymase of yeast or enzymes of bacteria, which render it impossible to extract except under special conditions and high pressures. I have concluded that such is the case in luminous bacteria and that this explains the absence of a luciferin-luciferase reaction in these forms.<sup>2</sup> It seems possible also, however,

<sup>2</sup> Harvey, E. N., *Am. J. Physiol.*, 1916, xli, 449.

that very little luminous material is present in these forms at any one time, but that it is manufactured continuously by the living bacterial cell.

But in addition to the forms which may contain only small amounts of luciferin and luciferase, or unstable luciferin and luciferase, or luciferase in endoenzyme condition, there are at least two groups of animals which contain abundant luminous materials, whose light is long lasting, whose cells may be easily broken up, and in which the photogenic substances may be readily dried and give a bright light on again moistening. These are the medusæ, *Æquorea forskalea* and *Mitrocoma cellularia*, and the pennatulids, *Cavernularia haberi* and *Ptylosarcus* Sp.(?) especially the medusæ. *Æquorea* and *Mitrocoma*, found at Friday Harbor, Washington, contain many clumps of luminous cells about the rim of the umbrella at the base of the tentacles. Under the microscope masses of yellow material can be seen in the position from which the light comes, which probably are the photogenic cells. Gentle rubbing of the region liberates abundant luminous secretion which sticks to the fingers and which causes the sea water to luminesce quite brightly. The rim of the umbrella is easily cut away and this material, when squeezed through cheese cloth, gives a permanent bright luminous extract whose light lasts several hours. The animal itself luminesces only on stimulation.

There is, then, in these medusæ no lack of photogenic material. The material is readily extracted and stable, since the light lasts for several hours. Nevertheless, the luciferin and luciferase reaction cannot be obtained with these forms despite many attempts and care to guard against all sources of error.

What is the reason for this negative behavior? Is luminescence of jellyfish quite a different process from that in *Cypridina*, *Pholas*, or fireflies, which do give the luciferin-luciferase reaction? Or is the amount of luciferase in these forms just sufficient to oxidize the luciferin which is present and leave no excess in the extract? In *Cypridina* there is enough luciferase in one animal to oxidize the luciferin of 100 animals, but not an indefinite amount. *Cypridina* luciferase behaves as an enzyme but is not a perfect example of a catalyst which should transform indefinite amounts of substrate. There are, however, enzyme-like bodies known, the peroxidases of plants, in which there is a definite



mass relation between peroxidase and body oxidized, and it is not impossible that some luciferases behave in this way.

It should be pointed out in this connection that the light of these jellyfish comes unquestionably from granules of relatively large size. They can be seen at night under the microscope as dots of light with definite boundaries, not merely points of light. These granules shine brightly for some time, but if saponin or sodium glycocholate or fresh water is added, the granules dissolve with a sudden flare of light and then become dark. It is possible that the granule represents a combination of luciferin and luciferase in just the proper proportions for utilization. In *Cypridina* no such luminous granules exist in the extract (although granules occur in the luminous gland) or if they do exist in the extract they are ultra-microscopic in size.

It should also be borne in mind that Harden and Young<sup>3</sup> found an excess of zymase proper in some yeasts and an excess of co-zymase in other kinds. There is a certain resemblance between the luciferin-luciferase complex and the co-zymase-zymase complex and we may have an excess of luciferin in some animals and an excess of luciferase in others. Only if the latter condition existed could we demonstrate the presence of these two bodies.

I had hoped to solve this question by determining if the luciferin of medusæ will give light with the luciferase of some other form; *i.e.*, with a solution which we know to contain luciferase, as that of *Cypridina*. Such a test has given absolutely negative results. The luciferin of *Æquorea*, *Mitrocoma*, *Cavernularia* or *Ptylosarcus*, prepared in various ways, will give no light with *Cypridina* luciferase. Neither will the reverse "cross" (*Medusa* luciferase and *Cypridina* luciferin) give luminescence. These results are given in Table II.

This would seem to indicate that there was no *Mitrocoma* nor *Æquorea* nor pennatulid luciferin in the extract. There is a possibility, however, that *Cypridina* luciferase is absolutely specific and will not act with the luciferins of other forms. If that is the case, and my work shows that luciferin and luciferase are specific, except for very closely related forms, we cannot expect to throw light on the problem by this method.

<sup>3</sup> Harden, A., and Young, W. J., *Proc. Roy. Soc. Biol.*, 1906, lxxvii, 405; lxxviii, 369

There is a second method of attacking the problem. Suppose we prepare a solution which should contain medusa luciferin. On adding this to a glowing extract of medusæ, which must contain luciferin and also luciferase to oxidize the luciferin, a brighter light should result because within certain limits, with a given amount of luciferase, the more luciferin is present, the brighter will be the light. Trials have shown that no brighter light results from adding additional medusæ luciferin to a glowing medusa extract. Apparently, therefore, the medusa luciferin solution contains no luciferin or the glowing extract of medusæ contains no luciferase; in other words, these substances do

TABLE II.

"Cross."				Reaction.
<i>Mitrocoma</i> luciferase	+	<i>Mitrocoma</i> luciferin.....		Negative.
"	"	+ <i>Cypridina</i> "		"
<i>Æquorea</i>	"	+ <i>Æquorea</i> "		"
"	"	+ <i>Cypridina</i> "		"
<i>Cypridina</i>	"	+ <i>Mitrocoma</i> "		"
"	"	+ <i>Æquorea</i> "		"
<i>Cavernularia</i>	"	+ <i>Cavernularia</i> "		"
"	"	+ <i>Cypridina</i> "		"
<i>Cypridina</i>	"	+ <i>Cavernularia</i> "		"
<i>Ptylosarcus</i>	"	+ <i>Ptylosarcus</i> "		"
"	"	+ <i>Cypridina</i> "		"
<i>Cypridina</i>	"	+ <i>Ptylosarcus</i> "		"
"	"	+ <i>Cypridina</i> "		Brilliant light.

not exist in the medusæ. While my work thus far points to this conclusion, we should certainly expect so fundamental a reaction as that of luciferin with luciferase to be universal. The statement that luciferin and luciferase do not occur in medusæ must therefore be considered as tentative and dependent on the present state of our knowledge.

#### *Specificity of Luciferin and Luciferase.*

The specificity of luciferin and luciferase is of considerable interest apart from the question discussed above. Accordingly, I have made a study of the luminescence resulting when *Cypridina* luciferin and luciferase is mixed with these bodies prepared from other animals of

TABLE III.

Organism.	"Cross."				Reaction.
<i>Bacteria.</i>	<i>Cypridina</i>	luciferase	+ <i>Bacteria</i>	luciferin.	Not tried.
	"	luciferin	+ <i>Bacteria</i>	luciferase.	" "
	<i>Bacteria</i>	luciferin	+ "	luciferase.	Negative.
<i>Cystoflagellates</i>	<i>Cypridina</i>	luciferase	+ <i>Noctiluca</i>	luciferin.	Negative.
	"	luciferin	+ "	luciferase.	"
	<i>Noctiluca</i>	luciferin	+ "	luciferase.	"
<i>Medusæ.</i>	<i>Cypridina</i>	luciferase	+ <i>Æquorea</i>	luciferin.	Negative.
	"	luciferin	+ "	luciferase.	"
	<i>Æquorea</i>	luciferin	+ "	luciferase.	"
	<i>Cypridina</i>	luciferase	+ <i>Mitrocoma</i>	luciferin.	"
	"	luciferin	+ "	luciferase.	"
	<i>Mitrocoma</i>	luciferin	+ "	luciferase.	"
<i>Pennatulids.</i>	<i>Cypridina</i>	luciferase	+ <i>Pennatula</i>	luciferin.	Negative.
	"	luciferin	+ "	luciferase.	"
	<i>Pennatula</i>	luciferin	+ "	luciferase.	"
	<i>Cypridina</i>	luciferase	+ <i>Cavernularia</i>	luciferin.	"
	"	luciferin	+ "	luciferase.	"
	<i>Cavernularia</i>	luciferin	+ "	luciferase.	"
	<i>Cypridina</i>	luciferase	+ <i>Ptylosarcus</i>	luciferin.	"
	"	luciferin	+ "	luciferase.	"
	<i>Ptylosarcus</i>	luciferin	+ "	luciferase.	"
<i>Ctenophores.</i>	<i>Cypridina</i>	luciferase	+ <i>Bolina</i>	luciferin.	Negative.
	"	luciferin	+ "	luciferase.	"
	<i>Bolina</i>	luciferin	+ "	luciferase.	"
<i>Annelids.</i>	<i>Cypridina</i>	luciferase	+ <i>Tomopteris</i>	luciferin.	Not tried.
	"	luciferin	+ "	luciferase.	Positive ?
	<i>Tomopteris</i>	luciferin	+ "	luciferase.	Not tried.
	<i>Cypridina</i>	luciferase	+ <i>Odontosyllis</i>	luciferin.	Negative.*
	"	luciferin	+ "	luciferase.	" *
	<i>Odontosyllis</i>	luciferin	+ "	luciferase.	Positive.
	<i>Cypridina</i>	luciferase	+ <i>Polynöe</i>	luciferin.	Negative.
	"	luciferin	+ "	luciferase.	"
	<i>Polynöe</i>	luciferin	+ "	luciferase.	"
	<i>Cypridina</i>	luciferase	+ <i>Chatopterus</i>	luciferin.	"
	"	luciferin	+ "	luciferase.	"
	<i>Chatopterus</i>	luciferin	+ "	luciferase.	"
<i>Crustacea.</i>	<i>Cypridina</i>	luciferase	+ <i>Pyrocypis</i>	luciferin.	Positive.
	"	luciferin	+ "	luciferase.	"
	<i>Pyrocypis</i>	luciferin	+ "	luciferase.	"

\* Perhaps a slight reaction.

TABLE III—*Continued.*

Organism.	"Cross."				Reaction.
<i>Crustacea.</i>	<i>Cypridina</i>	luciferase	+	<i>Meganactiphanes</i> luciferin.	Negative.
	"	luciferin	+	" luciferase.	" "
	<i>Meganactiphanes</i>	luciferin	+	" luciferase.	" *
<i>Myriapods.</i>	<i>Cypridina</i>	luciferase	+	<i>Geophilus</i> luciferin.	Not tried.
	"	luciferin	+	" luciferase.	" "
	<i>Geophilus</i>	luciferin	+	" luciferase.	Negative (rather dilute solutions).
<i>Insects.</i>	<i>Cypridina</i>	luciferase	+	<i>Luciola</i> luciferin.	Negative.
	"	luciferin	+	" luciferase.	" "
	<i>Luciola</i>	luciferin	+	" luciferase.	Positive.
<i>Molluscs.</i>	<i>Cypridina</i>	luciferase	+	<i>Pholas</i> luciferin.	Negative.
	"	luciferin	+	" luciferase.	" (?)
	<i>Pholas</i>	luciferin	+	" luciferase.	Positive.
<i>Ascidians.</i>	<i>Cypridina</i>	luciferase	+	<i>Pyrosoma</i> luciferin.	Negative.
	"	luciferin	+	" luciferase.	" "
	<i>Pyrosoma</i>	luciferin	+	" luciferase.	" "
<i>Fish.</i>	<i>Cypridina</i>	luciferase	+	<i>Photoblepharon</i> luciferin.	Negative.
	"	luciferin	+	" luciferase.	" "
	<i>Photoblepharon</i>	luciferin	+	" luciferase.	" "
	<i>Cypridina</i>	luciferase	+	<i>Anomalops</i> luciferin.	" "
	"	luciferin	+	" luciferase.	" "
	<i>Anomalops</i>	luciferin	+	" luciferase.	" "

all degrees of relationship as regards *Cypridina*. The results are best expressed in the form of a tabulation. In this table the words "luciferin" and "luciferase" are used to indicate extracts so prepared that they should contain luciferin and luciferase, provided these substances are formed by the organism in question. Table III represents the results of experiments performed at various times during the past seven years in all parts of the world. Its incompleteness is due to the difficulty of obtaining luminous animals in sufficient quantity for the test in question.

From the evidence in Table III it would seem that *Cypridina* luciferase will only give light with the luciferin of a very closely related form, *Pyrocypriis*, a genus that differs from *Cypridina* only in the character of appendages on the upper lip. Both genera belong to the family, *Cypridinidæ*. Crosses of *Cypridina* luciferase with the luciferin of *Odontosyllis*, *Pholas*, or fireflies, all of which possess a luciferin-luciferase reaction, gives only negative or very faint positive results as does the reverse mixture, *Cypridina* luciferin crossed with the luciferase of these other forms. All my attempts to oxidize *Cypridina* luciferin with oxidizing enzymes or oxidizing agents of various kinds have failed, and also all attempts to oxidize with light production, various easily oxidizable substances with *Cypridina* luciferase.<sup>4</sup> We must therefore conclude that these two substances responsible for the production of light by *Cypridina*, are specific to the highest degree.

#### SUMMARY.

Among sixteen groups of luminous forms investigated by the author, in only four (fireflies, *Pholas*, ostracods, and *Odontosyllis*) is it possible to demonstrate the luciferin-luciferase reaction. In many groups this is probably due to the small amount of these substances present in the luminescent organism or to their instability. In the medusæ and pennatulids, despite a large amount of luminescent material, luciferin and luciferase cannot be demonstrated. This does not appear to be due to the presence of luciferin and luciferase in equivalent proportion, or to their instability. In fact, one is led to the conclusion that luciferin and luciferase do not exist in these forms, but such a conclusion must be regarded as merely tentative, in view of the fundamental character of the luciferin-luciferase reaction. Luciferin of one form will not luminesce with the luciferase of another form or *vice versa*, unless very closely related (*Cypridina* and *Pyrocypriis*). All experiments emphasize the specificity of the light producing substances of *Cypridina*.

<sup>4</sup> Harvey, E. N., *J. Gen. Physiol.*, 1918-19, i, 269.



# THE EFFECTS OF RADIUM RAYS ON METABOLISM AND GROWTH IN SEEDS.

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The view is current in the literature on the physiological action of radium radiations that these rays produce their effects by altering the metabolism of the tissues exposed to them. The basis for this view appears to be the observation that exposure to radium changes the rate of cell division and growth. If the term metabolism is to be used in describing the reaction of organized matter to any reagent, in order to attain analytical significance it must be defined in terms of some definite set of chemical transformations. We have consequently carried out a series of experiments designed to test the effect of the  $\beta$ -rays of radium on those metabolic processes which result in the production of  $\text{CO}_2$  and to demonstrate whether any correlation exists between alterations produced in these activities and the ability of the protoplasm to take part in cell division and growth.

It is essential for such an experiment to select material which will enter into some well defined growth process, and of which the  $\text{CO}_2$  production can be examined before any change in the bulk of protoplasm has brought about a significant alteration in the quantity of  $\text{CO}_2$  produced. For this purpose we have made use of radish seeds, and have measured the  $\text{CO}_2$  given off, before germination has had time to take place. Doubtless, cell division and growth were beginning to take place while the measurements were being made, but any changes in  $\text{CO}_2$  production which occurred as a result were not sufficient to mask the effects produced by the radiations.

The output of  $\text{CO}_2$  was measured in the apparatus described by Osterhout.<sup>1</sup> Fifty or seventy seeds were moistened and spread over the surface of a test-tube. Air was circulated between this test-tube

<sup>1</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1918-19, i, 17.

and another containing dilute NaOH and phenol red, and a measurement was made of the time required for the liberated  $\text{CO}_2$  to change the reaction of this solution from pH 7.60 to pH 7.09. The rate of  $\text{CO}_2$  production per seed was expressed by the reciprocal of this time divided by the number of seeds.<sup>2</sup> During the measurement the tube containing the seeds was immersed in a water bath at constant temperature, usually at 25°C.

In order to determine the accuracy of the method, and the variations which would result from measuring different groups of seeds a series of control experiments was performed. In each the seeds were divided into two numerically equal lots, and six or eight successive measurements made of the  $\text{CO}_2$  production. Table I shows that the

TABLE I.  
*Comparison of Unradiated Seeds.*

No. of seeds per lot.	CO <sub>2</sub> production per seed.*		Difference.
	Lot 1.	Lot 2	
			<i>per cent</i>
50	630 ± 6.8	610 ± 11.0	± 3.1
50	696 ± 4.3	782 ± 10.7	± 11.0
50	1516 ± 18	1470 ± 20	± 3.2
50	286 ± 2.5	252 ± 2.6	± 13.4
70	480 ± 2.67	354 ± 2.83	± 35.6
70	458 ± 5.9	422 ± 13.8	± 8.4

\* Method of calculating explained in the text.

probable error of the mean of the successive measurements on any lot does not exceed as a rule 5 per cent. The percentage difference in measurements of different lots is less than 13 per cent in all but one of the experiments.

The ability of the seeds to germinate and grow was examined by placing comparable lots of seeds on moist filter paper. They were covered by a large glass vessel to prevent rapid drying, but access of fresh air was not completely cut off. It was found that at least 95 per cent of unirradiated seeds germinated and grew under these conditions.

<sup>2</sup> The seeds were weighed in the dry condition and the  $\text{CO}_2$  production estimated per gram of seeds. The relative  $\text{CO}_2$  productions of different lots of seeds expressed in this way did not differ materially from those obtained by the method employed above.



The seeds were exposed to the  $\beta$  and  $\gamma$ -rays of radium by placing them in the dry condition in a  $\frac{1}{8}$  inch tube, closely packed about a glass tube containing radium emanation.<sup>3</sup> The  $\alpha$ -rays failed to penetrate the walls of the tube; in comparison to the  $\beta$ -rays, the  $\gamma$ -rays doubtless contributed to the effect in negligible degree owing to their limited absorption in the seed. 2 days after radiation the seeds were moistened and the  $\text{CO}_2$  production was determined. They were then given an opportunity to germinate and grow. In each experiment an equal number of unirradiated seeds was kept as a control. Their  $\text{CO}_2$  production was measured and they were then set to germinate on the same moist filter paper and under the same cover with the radiated seeds.

TABLE II.  
*Comparison between Radiated and Unradiated Seeds.*

No. of seeds per lot.	CO <sub>2</sub> production per seed.*		Difference.	Amount germinated on 3rd day.	
	Radiated.	Control.		Radiated.	Control.
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
54	851	748	+ 13.8	26	59
56	877	777	+ 12.9	19	81
50	753	544	+ 38.4	28	100
50	696	445	+ 56.2	16	72
70	445	370	+ 20.4	40	90
70	360	216	+ 66.5	23	98

\* Method of calculating explained in text.

Table II, containing the results of six experiments, shows that the difference in the rate of  $\text{CO}_2$  production between radiated and unirradiated seeds is greater in almost every case than that found between comparable lots of unirradiated seeds, and that *the rate of  $\text{CO}_2$  production in radiated seeds is invariably greater than in the corresponding unirradiated control.* In comparison to this it is seen that the germination of the radiated seeds is retarded to a marked degree. A notable feature is that the radiated seeds germinate much more slowly than normal for it usually requires 2 days before any of these seeds send out roots, and the number of seeds which have done so increases from day

<sup>3</sup> We are indebted to Dr. William Duane for placing a supply of radium belonging to the Cancer Commission of Harvard University at our disposal.

to day much more slowly than in the case of the controls. In addition, many seeds seem to be permanently prevented from germinating, so that at the end of a week the number which have commenced to grow is far below that found in a lot of unirradiated seeds. The seeds which fail to germinate as the result of radiation are not killed, as is shown by the undiminished  $\text{CO}_2$  production. Twenty-five radiated seeds from the radiated lot described in the fourth experiment of Table II which had failed to germinate after 7 days still produced  $\text{CO}_2$  at a rate of 606 units per seed.

These results indicate that no direct relation exists between the effect of  $\beta$ -rays on  $\text{CO}_2$  metabolism and growth in radish seeds. In this respect they recall the observation of Warburg<sup>4</sup> and Loeb and Wasteneys,<sup>5</sup> that by decreasing the hydrogen ion concentration of sea water, the  $\text{O}_2$  absorption of echinoderm eggs increases and at the same time segmentation is prevented.

Kimura<sup>6</sup> has shown that if living carcinoma and sarcoma cells from the mouse are exposed *in vitro* to weak doses of x-rays the processes of oxidation which lead to  $\text{CO}_2$  production are stimulated and that in the case of the sarcoma the growing power is increased to some extent. Stronger radiation diminishes the  $\text{CO}_2$  production of both types of cell and destroys their power of mitotic division and growth. The important point which is demonstrated by the present experiments, in contrast to those of Kimura, is that the *changes in the rates of  $\text{CO}_2$  production and cell division do not always go hand in hand; one may be increased by exposures which retard the other.* This conclusion is not as unexpected as it may appear at first, for the pathological changes which are produced in living matter by radiations, *i.e.* the malformation of embryos, must be due to an unequal effect on various processes which go on side by side. If all processes were affected alike their courses might be run more rapidly or more slowly, but at any instant the conditions of equilibrium in the tissue would not be abnormal. It is the specific action of radiations on certain physiological processes in contrast to others which accounts for the characteristics of their effects.

<sup>4</sup> Warburg, O., *Z. Physiol. Chem.*, 1910, lxxi, 305.

<sup>5</sup> Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1913, xiv, 355.

<sup>6</sup> Kimura, N., *J. Cancer Res.*, 1919, iv, 95.

It is because radiations have a specific action on certain physiological processes in contrast to others that their characteristic effects are produced. Consequently it is unjustifiable to make any general assumption concerning their action on the chemical changes of living matter as a whole.



# CORRESPONDENCE OF SKIN PIGMENTS IN RELATED SPECIES OF NUDIBRANCHS.

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(Received for publication, October 21, 1921.)

That pigmentary materials occurring in various species of certain genera of marine invertebrates may be chemically very similar, or even identical, has several times been suggested.<sup>1</sup> In some cases the presence of such chemically similar coloring matters, playing an important rôle in determining the appearance of the animals, has a distinct part in the theory of animal coloration, since, if substantiated, identity of composition as regards pigments may be significant for the evolutionary origin of types of pigmentation (*e.g.* in so called "warning" coloration<sup>2</sup>). The pigmentation of the large nudibranch *Chromodoris zebra* has been studied from the standpoint of its possible adaptive quality,<sup>3</sup> and since it happens that coloring matters superficially corresponding to those in *Chromodoris zebra* are found in a good many species of this widely dispersed genus,<sup>4</sup> it is desirable to examine the degree of chemical similarity of these substances. The blue pigment which plays a conspicuous part in the coloration of these creatures has properties favorable for such study. A yellow pigment, also of frequent occurrence in the genus, belongs to the carotinoid "lipochrome" series, but because of the very general occurrence of such substances as pigmentary components derived from plant food, it is less significant for the present purpose.

<sup>1</sup> Briot, A., *Compt. rend. Soc. Biol.*, 1906, lxxxviii, 1157. Crozier, W. J., *Zool. Jahrb., Abt. Zool.*, 1915, xxxv, 233; *J. Biol. Chem.*, 1918, xxxv, 455; *Am. Naturalist*, lii, 552.

<sup>2</sup> Crozier, W. J., *Anat. Rec.*, 1919-20, vii, 349.

<sup>3</sup> Crozier, W. J., *Anat. Rec.*, 1919-20, vii, 349; *Proc. Nat. Acad. Sci.*, 1916, ii, 672.

<sup>4</sup> Crozier, W. J., *J. Physiol.*, 1913-14, xlvii, 491.

Solutions of the blue material were compared in the case of the three species: *Chromodoris zebra* from Bermuda, *Chromodoris porterae* from La Jolla, California, and *Chromodoris universitalis* from Laguna Beach, California. An extract from specimens of any of these species in aqueous formaldehyde, is bluish purple in color, turned pink by acids at about pH 5.6, and changed to blue (with some precipitation of a greenish flocculent material) by alkali.<sup>5</sup> In some other qualitative respects the substances extracted from the three species also agree, notably in yielding positive tests for the presence of manganese, and in being decolorized, reversibly reduced, by putrefactive bacteria; but the most important data are derived from the absorption spectra. In formaldehyde solution the *Chromodoris zebra* pigment gives a narrow, sharply bounded absorption band centering on 620 to 622 $\mu$ . In alcoholic extract, neutral or faintly acid, the absorption band likewise centers at 622 $\mu$ .

The extracts are stable for at least several years (especially if shielded from bright sunlight), hence it is unnecessary to employ freshly prepared solutions of the pigments. *Chromodoris porterae* pigment, in the comparison spectroscope, gave an absorption which could not be distinguished from that of *Chromodoris zebra*, and, as determined by successive dilutions, the absorption maximum was also at about 620 $\mu$ . This is likewise true of the pigment of *Chromodoris universitalis*.

It is believed, consequently, that the blue pigment which has a special importance for the coloration of the genus *Chromodoris* is a very similar body, chemically, in widely dispersed species of this genus;<sup>6</sup> and since the habits of the various species differ considerably, it must be reckoned as a distinct probability, therefore, that the pigment has primarily no relation to the habits of these animals, but is an expression, merely, of the fundamental chemistry of the Chromodorid stock.

<sup>5</sup> cf. Crozier, W. J., 1916, *J. Biol. Chem.*, xxiv, 255. In alcoholic solution the green material is soluble.

<sup>6</sup> *Chromodoris porterae* were obtained from the Scripps Institution, and *Chromodoris porterae* and *Chromodoris universitalis* from Professor W. A. Hilton of Pomona College; the author is grateful for this assistance.

# THE PHYSIOLOGY OF THE RESPIRATION OF FISHES IN RELATION TO THE HYDROGEN ION CONCENTRATION OF THE MEDIUM.\*

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## INTRODUCTION.

Within recent years much has been added to the knowledge concerning the mechanism of the respiratory function of the blood. Haldane and Priestley (1905) have shown that, at least in the higher animals, the respiratory movements are affected by the carbon dioxide tension of the arterial blood. It has been shown definitely (Hasselbalch, 1912 and citations) that the exciting agent is the hydrogen ion concentration of the blood bathing the respiratory center.

Krogh and Leitch (1919) undertook to study the respiratory function of the blood of fishes in view of the knowledge of the influence of temperature upon the dissociation curve of oxyhemoglobin as investigated by Barcroft and Hill (1909) and Barcroft (1914). These workers found that the blood of the fish was especially adapted to its needs.

Certain marine fishes are known to react to a gradient of acidity and alkalinity (Shelford and Powers, 1915). It has been found that certain species react positively to a definite range of hydrogen ion concentration of the sea water, others are less definite in their reaction, and still others seemingly do not respond to differences in alkalinity and acidity (Powers, 1921).

In view of these facts experiments were undertaken to determine the ability of marine fishes to extract oxygen from the sea water at different hydrogen ion concentrations. Interest in this question was

\* Studies from The University of Nebraska, No 131.

further stimulated by the theory held by Roule (1915) that the salmon (*Salmo salar* L.) does not respond to salinity or temperature but that it always reacts in such a way as to bring it into water having a higher oxygen content.

*Method.*

A very simple technique was employed. A fish was placed in a 2 quart Mason jar filled with sea water and made air-tight with a rubber stopper. The jar was then immersed in a water bath of running sea water in which the temperature was almost constant. The oxygen content of the water at the beginning of an experiment was always sufficiently high so that the fish did not at first suffer from oxygen want. The hydrogen ion concentration was determined by the colorimetric method; and the oxygen, by the Winkler method immediately after all movements of the fish had ceased.

The hydrogen ion concentration of the sea water was varied by aerating with carbon dioxide-free air or by the addition of a small amount of sea water made alkaline by the addition of sodium hydroxide and by the introduction of carbon dioxide.

The fishes used in these experiments were kept in a small aquarium of running sea water. They were allowed to rest from 6 to 12 hours, so that they might recover from any shock suffered when collected. All were rejected after being in the laboratory 2 or 3 days so as to avoid as far as possible erratic results due to the ill effect of keeping them under unnatural conditions.

EXPERIMENTAL.

The mean oxygen content of the sea water of all experiments having approximately the same pH at the time of death of the fish was calculated. That is, if the highest oxygen content of the sea water at the time of death of the cunner at 7.22 to 7.25 pH was 0.30 cc. per liter, and the lowest 0.20 cc. per liter, the cunner was taken to be able to absorb the oxygen from the sea water down to 0.25 cc. per liter at a pH from 7.22 to 7.25. This was taken to be more nearly exact and to eliminate to a greater extent the factor of individual variation than if individuals were taken alone. Loeb (1912) has shown that the mortality curve of *Fundulus* embryos under the in-



fluence of acids, alkalies, and potassium salts, and Loeb and Northrop (1917) that the mortality curve of the fruit fly (*Drosophila*) under the influence of food and temperature are probability curves. The probable cause of the variations in the ability of different individual fish of the same species to absorb oxygen from the medium at low oxygen pressure at a given hydrogen ion concentration and temperature will be taken up later.

The figures are graphic representations of the experimental data.

*The Ability of Fishes to Extract Oxygen from the Sea Water at Different Hydrogen Ion Concentrations.*

(a) *The Cunner* (*Tautoglabrus adspersus* Walbaum).—The cunner was able to extract oxygen from the sea water down to approximately 0.25 cc. per liter before asphyxiation at all hydrogen ion concentrations up to about 6.5 pH. From this point on there seems to be a rapid loss in the ability of this fish to extract oxygen from the sea water at low oxygen pressure (Fig. 1).

(b) *The Butter-Fish* (*Poronotus triacanthus* Peck).—Fig. 2 shows that the butter-fish was not able to extract the oxygen from the sea water at quite as low a level as the cunner. The general trend of the curve indicates that the butter-fish is able to absorb oxygen at the lowest concentrations at a pH of about 7.6 to 7.8. This was shown even more strikingly when individuals were considered, the lowest point at which oxygen was absorbed being 0.36 cc. per liter at 7.58 pH and 0.35 cc. per liter at 7.57 pH. There was a slight falling off in the power to extract the oxygen at low concentrations, as the hydrogen ion concentration of the sea water was lowered from a pH of about 7.6 or 7.8 to 9.0. The falling off was much more rapid when the hydrogen ion concentration was increased from a pH of about 7.6 to 6.3, being most rapid from 7.1 to 6.35. At the higher hydrogen ion concentrations but very little of the oxygen had been absorbed before asphyxiation took place.

(c) *The Mackerel* (*Scomber scombrus* L.).—There was only a slight variation in the ability of the mackerel to extract oxygen from the sea water at low tension from about 7.7 pH to about 8.2 pH within an optimum at about 8.0 pH. There was a more or less falling off in

this ability when the pH of the water was either raised above or lowered below this range (Fig. 1).

(d) *The Alewife* (*Pomolobus pseudoharengus* Wilson).—The optimum pH for the absorption of oxygen at low oxygen tension for the

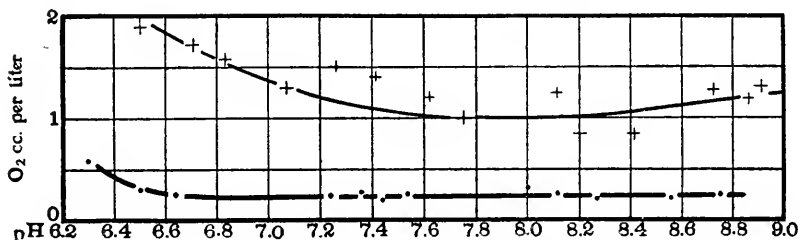


FIG. 1. The circles represent the experimental data of the cunner (*Tautoglabrus adspersus* Walbaum) and the plus signs those of the mackerel (*Scomber scombrus* L.). The abscissæ give the hydrogen ion concentration; and the ordinates the oxygen in cc. per liter at the end of the experiment.

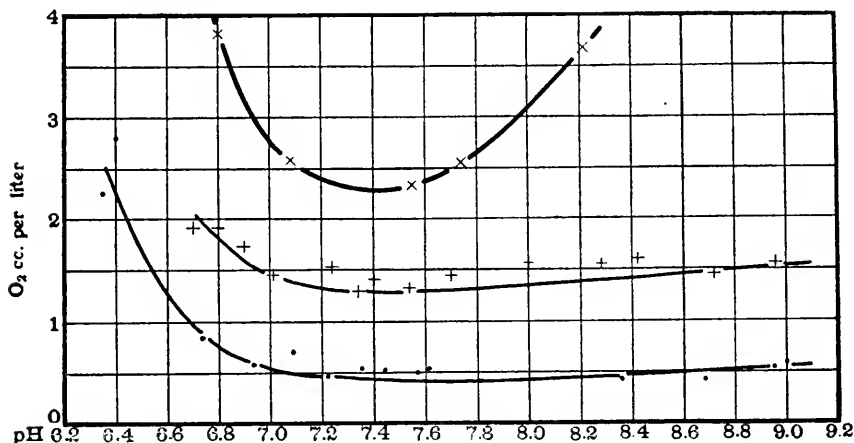


FIG. 2. The circles represent the experimental data of the butter-fish (*Poronotus triacanthus* Peck), the plus signs those of the alewife (*Pomolobus pseudoharengus* Wilson) and the crosses those of the herring (*Clupea harengus* L.). The abscissæ give the hydrogen ion concentration; and the ordinates the oxygen in cc. per liter at the end of the experiment.

alewife was about 7.3 or perhaps 7.3 to 7.6 (Fig. 2). The falling off from this optimum was greatest when the hydrogen ion concentration was raised above this optimum. The falling off was less marked when the hydrogen ion concentration was lowered.

(e) *The Herring* (*Clupea harengus* L.).—The herring (?) used in these experiments averaged from 3 to 4 gm. This fish appeared in great numbers for only a few days. Their sudden disappearance terminated the experiments and prevented the writer from having his identification of the species verified by a specialist. The young of the alewife were running at the same time and were taken by members of the U. S. Bureau of Fisheries.

The herring (Fig. 2) showed greater differences in ability to absorb oxygen at low oxygen tension with variation in the hydrogen ion concentration of the sea water than any of the other fishes tested. There was a very rapid falling off of the power to absorb oxygen from the sea water when the hydrogen ion concentration was raised at least above a pH of 7.4. It was less rapid when the hydrogen ion concentration was lowered.

The experiments just described show that the lowest tension at which oxygen can be absorbed from the sea water by certain marine fishes is more or less dependent upon the hydrogen ion concentration of the sea water. Of the five species of fishes tested, the cunner, butter-fish, mackerel, alewife, and herring, the cunner and butter-fish were affected least by a change in the hydrogen ion concentration. The lowest point at which the butter-fish was able to absorb oxygen from the sea water was at 0.35 cc. per liter at a pH of 7.57.<sup>1</sup> The mackerel, alewife, and herring were most affected by the variation in the pH of the sea water; *i.e.*, have the narrowest optimum pH. These fishes are considered the most sensitive and most restricted in their migratory movements. On the other hand, the cunner which is least affected is more cosmopolitan as to habitat and is an all year resident (Sumner, Osburn, and Cole, 1911). The butter-fish which shows less resistance to the variation in the pH is somewhat less cosmopolitan than the cunner in its habitat and is a resident of the Woods Hole vicinity only from early summer to late fall.

<sup>1</sup> This could not be called the optimum pH of the sea water for carrying on the respiratory functions by this fish. At the same time oxygen is being absorbed from the sea water bathing the gills, carbon dioxide is being given off. The pH at the beginning of the experiment was 8.28. Thus it stands to reason that the optimum pH of the sea water for this fish would be some where between 8.28 and 7.57.

When the figures are examined more closely it is seen that the optimum pH for the absorption of oxygen at low tension varies with the different species. The herring has the lowest pH optimum which seems to be correlated with the habits of this fish.

*Results of Experiments on the Pacific Herring (Clupea pallasii Cuv. and Val.).*

The foregoing experiments were performed at the Marine Biological Laboratory, Woods Hole, during the summer of 1920. It was thought desirable to test the Pacific herring, *Clupea pallasii*, in the same manner since its behavior was better known (Shelford and Powers, 1915 and Powers, 1921) than the Atlantic herring *Clupea harengus*. These experiments were performed at the Puget Sound Biological Station, Friday Harbor, during the summer of 1921. The methods employed in the experiments were similar to those at Woods Hole. In order to eliminate individual variation and make the time until death shorter, five 1.5 to 2.5 gm. fish were put in a 2 quart Mason jar of sea water instead of only one. The oxygen was determined immediately after the last fish had died. A total of three hundred and seventy-five fish were tested. Instead of taking the means of the extremes of the oxygen content at the end of an experiment, as was done in the preceding experiments, a curve, which is the mathematical mean of all experiments, was drawn (Fig. 3). The greatest deviation from the mean was 0.18 cc. per liter with the exception of three experiments. In two, one at 6.95 pH where the fish died when the oxygen content was 1.08 cc. per liter and another where the pH was 7.88 and the oxygen content was 0.98 cc. per liter at the end of the experiment, the variation from the mean was plus 0.25 cc. per liter. These two experiments seemed somewhat erratic but they have been included in all averages and calculations. In another, where the pH was 7.43, the variation from the mean was 0.20 cc. per liter. The average variations were plus 0.089 and minus 0.085 cc. per liter. This is less than 2 per cent of the total oxygen content of the sea water at the beginning of the experiments and less than 12 per cent of the difference of the extremes of the oxygen content of the sea water at the time of death of the fish. The

extreme variation from the mean is less than 33 per cent of the total variation in the experiments.

Duplicate oxygen tests could not be made by the methods employed since only 2 quarts of sea water were used in each experiment. However, the accuracy of the method was determined in connection with other work done at the same time. In 88 duplicate oxygen tests, the average deviation from the mean was  $\pm 0.024$  cc. per liter. This is more than 27 per cent of the average variation of the oxygen content at end of experiment from the mean curve (Fig. 3). The extreme

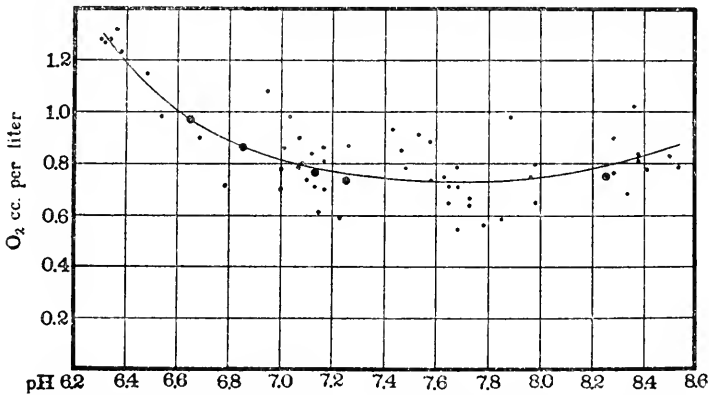


FIG. 3. The small circles represent the experimental data of the herring (*Clupea pallasii* Cuv. and Val.) when five fish were tested in a 2 quart mason jar, and the large circles when ten to twenty-five fish were tested in a 42 liter carboy. The abscissæ give the hydrogen ion concentration; and the ordinates the oxygen in cc. per liter at the end of the experiment.

deviations of the 88 duplicate tests were one 0.094 cc. per liter and four from 0.078 to 0.072 cc. per liter. This, again, is more than 33 per cent of the extreme deviations of the experiments from the mean curve. In other words, from 27 per cent to 33 per cent of the deviation of the experiments are probably due to the method employed and not the individual variation of the fish. Thus the average individual variation of the fish is less than 8 per cent and the extreme is less than 22 per cent of the difference of the total variations of the experiments. Further justifications for the form of the curve will be taken up in connection with the discussion of other experiments.

In order to determine whether or not the death of the fish was due to asphyxiation because of low oxygen tension of the sea water, the first set of experiments was checked by testing the fish where they would not suffer from oxygen want so quickly. Ten to twenty-five fish were placed in a carboy (capacity about 42 liters) of sea water at different hydrogen ion concentrations. The results of these experiments conform very markedly to the mean curve, although the time until death of the fish in the carboy was from 24 to 48 hours as compared with 1 hour and 23 minutes to 2 hours and 40 minutes when five fish were tested in the 2 quart Mason jars (Fig. 3). This shows that the fish died from asphyxiation due to low oxygen tension in the sea water and not to other causes.

The experiments performed at Friday Harbor corroborate in every way those done at Woods Hole. The results of these experiments conform strikingly with the behavior experiments and field observations made by the writer on the same fish during the summers of 1918 and 1919. The lowest tension at which the herring, *Clupea pallasii*, can extract oxygen from the sea water is when the hydrogen ion concentration is  $\pm 7.68$  pH. This fish reacts positive to sea water having a pH of from 7.68 to 7.73 and was found most abundantly in the sea water in the vicinity of the Puget Sound Biological Station that had a pH of about 7.73 to 7.76 (Powers, 1921).

#### DISCUSSION.

Workers on fish respiration have found that fishes are able to survive at rather low oxygen tension without any apparent ill effect (Powers, 1921, citations). Gaarder (1918) found that the actual oxygen consumption of an anesthetized carp was reduced only slightly, 0.62 cc. per kg. per minute, until the oxygen in solution in the water had been reduced from 15.4 cc. per liter to about 1.13 cc. per liter when the gills were artificially bathed with water at a constant rate. It is not known whether or not this slight falling off of oxygen consumption could have been corrected had the respiratory mechanism of the fish been free to respond normally. Packard (1905) showed that *Fundulus heteroclitus* injected with 5/16 M sodium carbonate were able to live longer in a liter flask of sea water

stopped tightly to exclude air than those not so injected. He also found that when the fish were injected with  $m/250$  to  $m/500$  solution of acetic acid, they did not live as long as the control fish. From these experiments Birge and Juday (1911) suggested that "if a fish possessed the power to alter the composition of its blood somewhat, it would be able to adapt itself to water which contains only a comparatively small amount of dissolved oxygen." Might it not be possible in these experiments, since the oxygen tension was lowered by the respiration of the fish, that the differences in survival time of the fish were due to the variations in their abilities to absorb oxygen from the sea water at low oxygen tension under the conditions of the experiment? That is, the fish whose blood was best adapted to absorb oxygen at low tension at the particular carbon dioxide tension of the experiment would survive longest. This ability would perhaps depend upon the alkaline reserve of the blood of the fish. That is, if the hemoglobin of the blood of all the fish of a given species had the same optimum pH to carry oxygen, the fish would be able to extract oxygen from the water at low oxygen tension in the direct order of the alkaline reserve of the blood at a carbon dioxide tension higher than the optimum and in the reverse order of the alkaline reserve in a carbon dioxide tension below the optimum. In these experiments the carbon dioxide tension would tend to increase above the optimum for the absorption of oxygen at low tension since ordinary sea water was used and, as the oxygen was absorbed by the confined fish, carbon dioxide would be given off.<sup>2</sup>

The experiments recorded in the figures show that the species of fish tested have an optimum pH of more or less narrow or wide range for absorbing oxygen from the sea water at low oxygen tension. The limiting factor is perhaps the carbon dioxide tension of the sea water. The pH of the sea water with a given alkaline reserve is dependent upon the carbon dioxide tension (Henderson and Cohn, 1916, and McClendon, 1917). The optimum carbon dioxide tension of the sea water for a species of fish as suggested above would perhaps depend upon the alkaline reserve and the optimum pH of its blood.

<sup>2</sup> The author has performed experiments which give strong evidence for this probability, which are reserved for further publication.

The individual variation of fish of a given species might depend upon the individual variation in the alkaline reserve of the blood.

These conclusions are further emphasized by FitzGerald's observations (1913, 1915), and recent experiments by Henderson (1919), Haggard and Henderson (1920), and Henderson (1920) on the part played by the alkaline reserve of the blood in the acclimatization to altitude. It might be further suggested that the species of fish having the greatest ability to vary the alkaline reserve of their blood would also have the greatest power to withstand a variation in carbon dioxide tension of the water. The rapidity of the change in the carbon dioxide tension which a fish would be able to withstand would depend upon the rapidity with which it is able to vary the alkaline reserve of its blood. In man (Henderson, 1920) this seems to be a rather slow process. This is a more logical explanation since the carbon dioxide and oxygen tension in the environment of the fish are not so intimately connected as with the air-breathing animals. But as a general rule when the oxygen content of the sea water is low the carbon dioxide is increased (Powers, 1920). However, further experiments are necessary to settle these points.

In the light of these experiments it is easily seen why Wells (1913) found that high carbon dioxide was more rapidly fatal to fishes than low oxygen and why fishes are able to sense out and detect variations in carbon dioxide tension more easily than the variations in the oxygen tension (Shelford and Allee, 1913). Bayliss (1918) states: " . . . if increase in carbon dioxide be prevented, as by respiration of pure nitrogen, a man may become unconscious before experiencing any unpleasant symptoms."

It is conceivable that the carbon dioxide tension of the water could be raised above the carbon dioxide tension of the tissue capillary blood of the fish. Under this condition the oxyhemoglobin would be reduced to hemoglobin in the capillaries of the gills of the fish rather than the hemoglobin being oxidised to oxyhemoglobin. The fish would then suffer oxygen want more quickly in water with a high carbon dioxide tension and ordinary oxygen tension than in water with a low carbon dioxide tension and a low oxygen tension. Thus the probabilities are that in Wells' experiments the high oxygen tension was not antagonistic to the high carbon dioxide tension, in



the true sense of the word, but that the oxygen tension was below on the one hand, and on the other it was above the tension at which hemoglobin could be oxidised to oxyhemoglobin in the presence of a high carbon dioxide tension.

#### SUMMARY.

1. The ability of marine fishes to absorb oxygen at low tension from the sea water is more or less dependent upon the hydrogen ion concentration of the water.

2. The ability of fishes to withstand wide variations in the range of hydrogen ion concentration of the sea water can be correlated with their habitats. The fishes that are most resistant to a wide variation in the hydrogen ion concentration are most cosmopolitan in their habitat. Those that are least resistant to a variation in the hydrogen ion concentration are the most restricted in their range of habitat.

3. There is a close correlation between the optimum condition of the sea water for the absorption of oxygen at low tension by the herring (*Clupea pallasii*), the condition of the sea water to which they react positive and that in which they are found most abundantly.

4. It is suggested that the variation in the ability to absorb oxygen at low tension at a given pH of individuals of a species is dependent upon the alkaline reserve of the blood of the individual fish.

The author wishes to thank Professor Frank R. Lillie, Director, and Mr. G. M. Gray, Curator, of the Marine Biological Laboratory, and Professor T. C. Frye, Director of the Puget Sound Biological Station, for rooms, equipments, and materials for this work, and for many courtesies during its progress. The author wishes further to thank Professor Jacques Loeb for suggestions in the presentation of data, and Dean R. A. Lyman for reading manuscript.

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## THE EFFECT OF IODINE AND IODOTHYRIN ON THE LARVÆ OF SALAMANDERS.

### IV. THE RÔLE OF IODINE IN THE INHIBITION OF THE METAMORPHOSIS OF THYMUS-FED SALAMANDERS.

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In previous papers I have shown that feeding thymus gland to the larvæ of salamanders retards or completely inhibits metamorphosis<sup>1,2</sup> as well as growth.<sup>3</sup> It was also demonstrated<sup>2</sup> that the inhibition of both growth and metamorphosis is not caused by specific growth-inhibiting substances contained in the thymus, but by a deficiency of the thymus in certain substances which are necessary for growth and metamorphosis. Later I suggested that these substances might be identical with iodine.<sup>4</sup>

Recently, however, I found<sup>5</sup> that inorganic iodine even when administered in excessive quantities does not accelerate either growth or metamorphosis of salamander larvæ. Although this result does not necessarily mean that salamander larvæ can grow and metamorphose in the complete absence of iodine, the correctness of my assumption that inhibition of growth and metamorphosis of thymus-fed salamander larvæ may be caused by a deficiency of the thymus in iodine, became doubtful, and special experiments were carried out to test this assumption. In the light of these experiments which will be briefly reported below, it appears that the substances which are deficient in the food of thymus-fed salamander larvæ are not identical with iodine.

<sup>1</sup> Uhlenhuth, E., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 37.

<sup>2</sup> Uhlenhuth, E., *J. Gen. Physiol.*, 1919, i, 305.

<sup>3</sup> Uhlenhuth, E., *J. Exp. Zool.*, 1918, xxv, 141.

<sup>4</sup> Uhlenhuth, E., *J. Gen. Physiol.*, 1919, i, 473.

<sup>5</sup> Uhlenhuth, E., *Endocrinology*, 1922 (In press); *Biol. Bull.*, 1922 (In press).

Of three series of larvæ of *Ambystoma maculatum* from the same brood, Series 1 was fed earthworms and kept in iodine-free water, Series 2 was fed thymus and kept in iodine-free water, Series 3 was fed thymus and kept in water to which, from the 26th day on, 1 drop of a 1/20 M solution of inorganic iodine per 1000 cc. of water had been added.

Growth began to decrease in both thymus-fed series at an age of 19 days and practically ceased at an age of 26 days. From this time on inorganic iodine was administered to Series 3 as stated above, but did not improve the growth of this series at all. At the 54th day both thymus-fed series were divided into two lots and, in order to make sure that the ineffectiveness of the iodine was not due to a loss of the ability to grow, earthworms were fed instead of thymus. The result was as follows: In Series 2 the larger and stronger larvæ were continued on thymus; they did not grow, and finally died at an age of 82 days. The smaller larvæ received earthworms instead of thymus; they immediately began to grow, reached a normal size and finally metamorphosed. In Series 3 to which the iodine was administered the smaller larvæ received earthworms instead of thymus; this change of the diet again resulted in vigorous growth and in metamorphosis. The larger larvæ were continued on thymus, but in spite of the administration of iodine did not grow at all, until, at the 82nd, day, earthworms were used as food instead of thymus. This diet again resulted in normal growth and metamorphosis.

The objection could be raised that the amount of iodine was too small to permit of growth and metamorphosis. It is possible that the concentration of iodine was not high enough to permit of normal growth and metamorphosis. If, however, a lack of iodine had anything to do with the inhibition of growth and metamorphosis, even the slightest amount of iodine should have produced an improvement. Since even no improvement could be obtained by iodine-administration, but immediately followed the administration of earthworms, the conclusion seems to be justified that the inhibition of growth and metamorphosis of thymus-fed salamander larvæ is not caused by a deficiency of the thymus in iodine, but by a deficiency in certain substances, as yet unknown, which are contained in the earthworm.

# THE INFLUENCE OF FEEDING THE ANTERIOR LOBE OF THE HYPOPHYSIS ON THE SIZE OF AMBYSTOMA TIGRINUM.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 19, 1921.)

In a previous article<sup>1</sup> I have shown that feeding the anterior lobe of the hypophysis of cattle to metamorphosed salamanders not only produces a far higher rate of growth than does the feeding of earth-worms, but causes the animals to grow beyond the known maximum size of the species. The purpose of the present paper is to report on the results of a continuation of the experiments on *Ambystoma tigrinum*.

## *Hypophysis-Fed Animals Compared with Normal (Worm-Fed) Animals.*

Figs. 1 and 2 show the animals of Experiment 1 at an age of 88 weeks. In Fig. 1 are shown the three worm-fed controls, in Fig. 2 the two hypophysis-fed animals. Total length, weight, and sex are indicated in the figure legends. In comparing the controls with the experimental animals it will be noticed that hypophysis-feeding resulted in a larger size than the feeding of normal food. Figs. 3 and 4 show that a similar relation between the controls and the hypophysis-fed animals has persisted up to the day that the animals reached an age of 132 weeks. Fig. 3 shows the worm-fed controls of Experiment 1 at an age of 132 weeks, Fig. 4 the hypophysis-fed male (the female died) at the same age. Comparison of the photographs as well as of the figures for size and weight demonstrates that the hypophysis-fed animal is still by far the largest one.

A second experiment gave the same result. Fig. 5 shows the controls of Experiment 2; Fig. 6, the hypophysis-fed animals of the same experiment at an age of 88 weeks. Again the hypophysis-fed animals

<sup>1</sup> Uhlenhuth, E., *J. Gen. Physiol.*, 1920-21, iii, 347

are much larger and heavier than the worm-fed controls. The hypophysis-fed animals were continued on a diet of anterior lobe and increased at the same rate as the hypophysis-fed animals of Experiment 1;

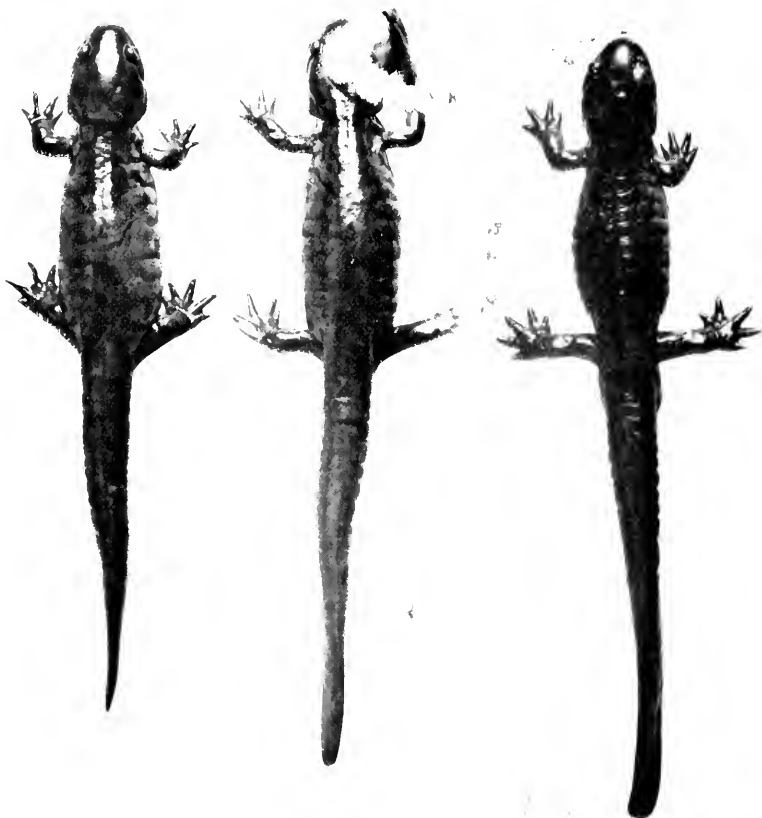


FIG. 1. Experiment 1. Worm-fed control animals at an age of 88 weeks. No. 1, female (total length, 177.5 mm.; weight, 34.8 gm.); No. 2, female (total length, 194.5 mm.; weight, 40.0 gm.); No. 3, male (total length, 200.5 mm.; weight, 35.0 gm.).

ment 1; they are shown in Fig. 7. All hypophysis-fed animals exceed the size of the largest known animal (235 mm.), the largest one being 25.1 per cent larger than the largest known animal. None of the worm-fed control animals has reached as yet the size of the largest known animal.



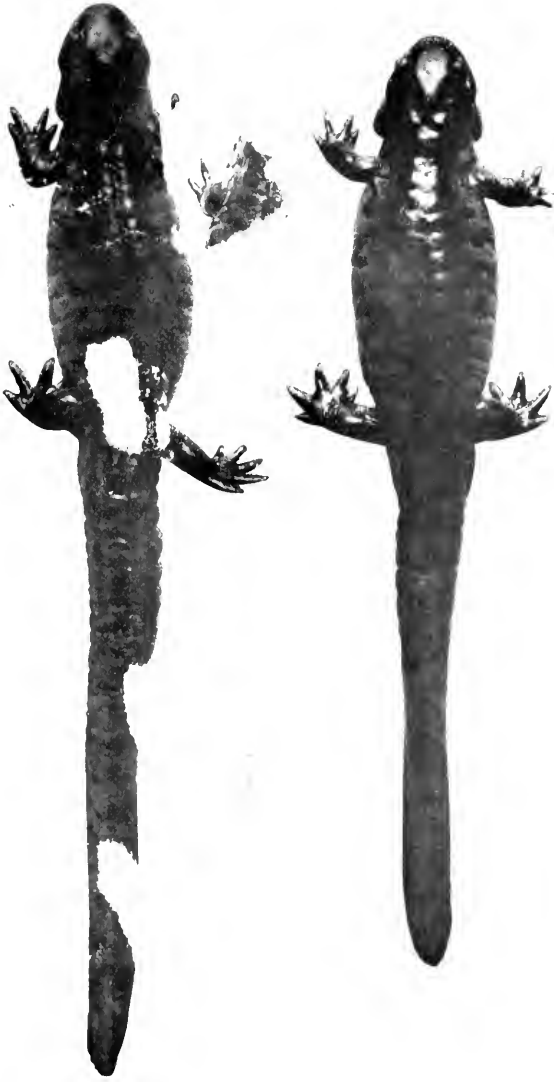


FIG. 2. Experiment 1. Anterior lobe-fed animals, at an age of 88 weeks. No. 4, male (total length, 273.5 mm.; weight, 72.3 gm.); No. 5, female (total length, 235.5 mm.; weight, 78.0 gm.).

*Hypophysis-Fed Animals Compared with Liver-Fed Animals.*

Several food substances besides earthworms had been tried out, but none of them gave higher rates of growth or produced larger animals

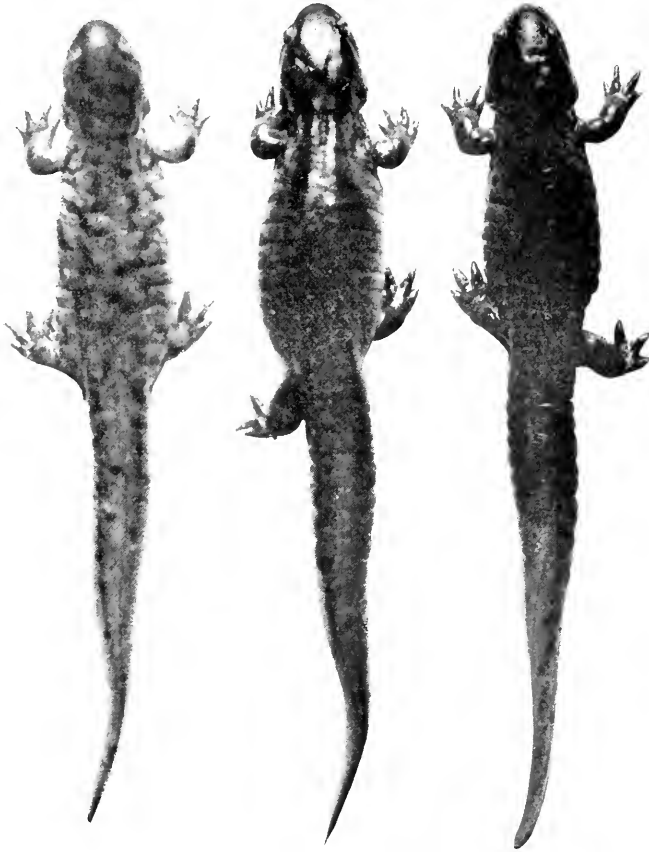


FIG. 3. Experiment 1. Worm-fed controls, at an age of 132 weeks. No. 1, female (total length, 200.8 mm.; weight, 57.0 gm.); No. 2, female (total length, 215.1 mm.; weight, 65.0 gm.); No. 3, male (total length, 212.0 mm.; weight, 55.0 gm.).

than did earthworms. Finally it was decided to use beef liver as food. The three worm-fed animals (Fig. 5) of Experiment 2 were used for this experiment. The two larger animals, male No. 1 and female No. 2, were fed liver, the smallest animal, male No. 3, was fed



FIG. 4. Experiment 1. Anterior lobe-fed animal, at an age of 132 weeks No. 4, male (total length, 294.0 mm.; weight, 108.0 gm.). No. 5 died; it measured 250.0 mm. when measured the last time (at an age of 126 weeks).

anterior lobe. The result is shown in Fig. 8. The hypophysis-fed male (to the right in Fig. 7), although it was by far the smallest animal before anterior lobe was fed (see Fig. 5), is now, after a period of ante-

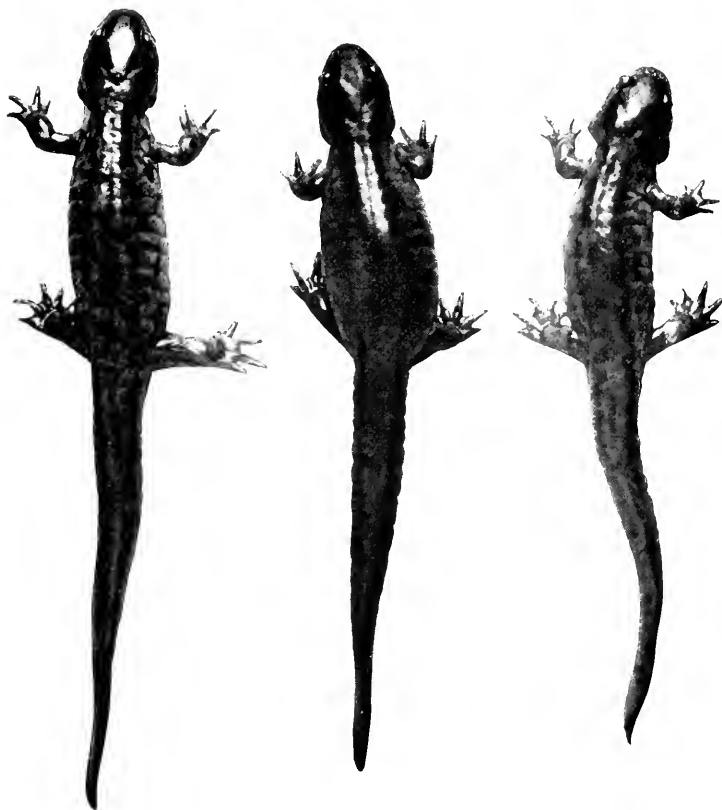


FIG. 5. Experiment 2. Worm-fed controls, at an age of 88 weeks. No. 1, male (total length, 204.1 mm.; weight, 38.5 gm.); No. 2, female (total length, 186.6 mm.; weight, 40.2 gm.); No. 3, male (total length, 181.5 mm.; weight, 31.1 gm.).

rior lobe-feeding of only 37 weeks, nearly just as large as, and heavier than the liver-fed control, male No. 1 (the difference in weight was less pronounced a week before but has increased during a period of starvation to which the animals were subjected in preparation for an operation). Moreover the liver-fed animals, although they were

growing in the beginning at the same rate as the hypophysis-fed animals, have stopped growing during the last 26 weeks, while the

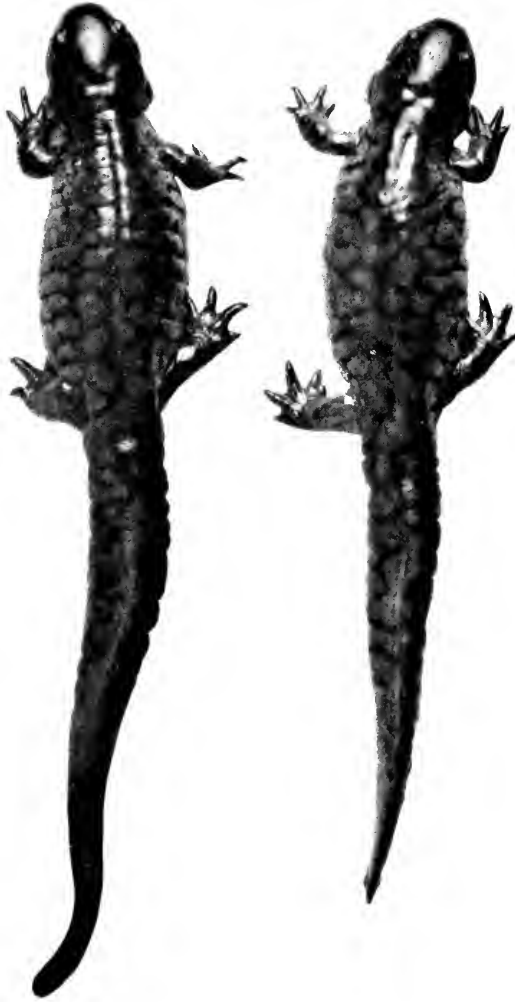


FIG. 6. Experiment 2. Anterior lobe-fed animals, at an age of 88 weeks; No. 4, male (total length, 257.1 mm.; weight, 95.0 gm.); No. 5, female (total length, 226.2 mm.; weight 84.3 gm.).

hypophysis-fed male, although it is of the same age and nearly of the same size as the liver-fed control, male No. 1, is still growing with



FIG. 7. Experiment 2. Anterior lobe-fed animals, at an age of 132 weeks; No. 4, male (total length, 285.0 mm.; weight, 108.0 gm.); No. 5, female (total length, 245.0 mm.; weight, 108.0 gm.).



FIG. 8. Experiment 2. Two of the controls fed liver and one fed anterior lobe for 37 weeks. No. 1, male (total length, 247.0 mm.; weight, 65.5 gm.); No. 2, female (total length, 212.0 mm.; weight, 61.0 gm.); No. 3, male (total length, 244.0 mm.; weight, 78.0 gm.; this animal was the smallest of the three before hypophysis-feeding started, as seen in Fig. 5).

considerable vigor. This condition may be taken as an indication that the hypophysis-fed animal will finally reach a size much in excess of the size of the liver-fed animals. The same result was obtained in two other experiments.

The liver diet not only permits a higher rate of growth than that resulting from an earthworm diet, but produces as high a rate of growth as that caused by anterior lobe-feeding. There is, however, an important difference between the effects of liver and hypophysis. The liver-fed animals stop growing a short while after liver-feeding begins, while the hypophysis-fed animals continue to grow at a high rate. That age and size have nothing to do with this difference, is clearly demonstrated by the fact that the hypophysis-fed male No. 3 of Experiment 2 is nearly as large (see Fig. 8) and just as old as the liver-fed male of the same experiment. The same result was obtained in two other experiments.

An inspection of the photographs shows that the liver-fed animals not only grow larger than the worm-fed animals, but exceed the known maximum size of normal animals of the species. This is the case with the liver-fed male, No. 1, of Experiment 2 (Fig. 8), which is 4.7 per cent larger than the largest known normal animal. It seems, however, that liver does not permit growth beyond a certain size far below that which hypophysis-fed animals attain.

#### SUMMARY.

1. Animals of the species *Ambystoma tigrinum* when fed anterior lobe can reach a size far in excess of that of animals fed earthworms and presumably also of that of liver-fed animals.

2. Liver produces a rate of growth as high as that resulting from anterior lobe-feeding, but maintains growth only, until the animals reach a definite size far below that of anterior lobe-fed animals.



## THE TEMPERATURE COEFFICIENT OF PHAGOCYTOSIS.

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Recently Madsen and Watabiki (1) have made some accurate measurements of the effect of temperature on the phagocytosis of bacteria. Fig. 1 is a reproduction of one of their figures showing the time curves of the number of bacteria ingested per leucocyte at different temperatures. In analyzing these results they endeavored to apply the familiar formula for a monomolecular reaction. In this formula,  $K = \frac{1}{T} \text{Log} \frac{A}{A - x}$ , they took  $A$  equal to the maximum number of bacteria ingested at the close of the experiment (instead of the maximum number of bacteria present, which, one is led to infer, was larger than  $A$  even at the higher temperatures) and  $x$  equal, as usual, to the number of bacteria ingested in time,  $T$ .

Considering their method of analysis it is not surprising that they found it impossible to calculate the temperature coefficient of phagocytosis from their figures. Inspection of Fig. 1 shows that the total number of bacteria ingested is smaller at the lower temperatures, *i.e.*,  $A$  is itself a function of temperature, increasing with rise of temperature. Now the accelerating effect of the higher temperatures is evidenced quite as much by the increase in  $A$  as by the increase in  $K$ , as they calculated it. The former factor they have completely discounted by their procedure, which is equivalent to "telescoping" the curves in Fig. 1 on the ordinate so that they all reach the maximum at the same point, and then comparing the times necessary for the different curves to reach the same ordinate. Moreover, the agreement of the experimental data with the formula for a monomolecular reaction was admittedly unsatisfactory at temperatures above 25°C. where the formula for a bimolecular reaction was usually found to give better results. But even if the results could be expressed

by the formula for a monomolecular reaction it could hardly be more than a meaningless coincidence. It could not indicate a rate proportional to the diminishing numbers of free bacteria because many bacteria were still present, at least at the lower temperatures, when phagocytosis had decreased to zero.<sup>1</sup> Nevertheless, the approximate empirical applicability of this formula to their results impressed the authors with the fact that phagocytosis obeys known physico-chemical

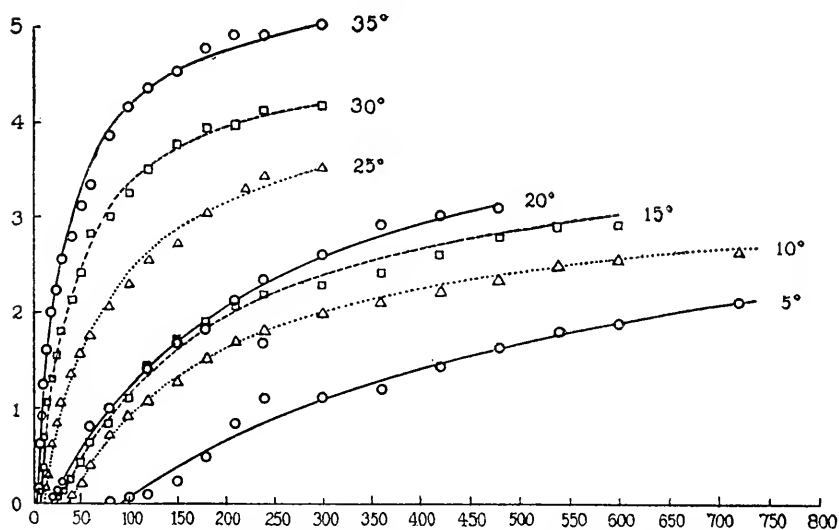


FIG. 1. Reproduction of curves from data of Madsen and Watabiki<sup>2</sup> showing the number of bacteria taken up per leucocyte (ordinates) as a function of time (abscissae) at different temperatures. Note the shorter latent period, higher maximum and shorter duration of the experiment at higher temperatures.

laws. Actually, the attempt to force the experimental results to fit a known formula has merely beclouded the issue. It is worth while trying whether a more rational analysis of these curves will yield a more significant result.

Osterhout (3), in discussing the analysis of time curves, has pointed

<sup>1</sup> In a previous paper on phagocytosis (2) the writer found that the rate of ingestion of solid particles by leucocytes was proportional to the decreasing number of available particles, thus giving a constant  $K$  when calculated by the formula for a monomolecular reaction.

out that one should compare the times necessary for equal amounts of action rather than the amount of action at equal times. This is equivalent to saying that the rates of two reactions should be compared at *corresponding stages*. In the curves of Fig. 1, however, the corresponding stages are not points of equal amount of action because the maximum varies at different temperatures, but, rather, they are points of equal percentages of the total amount of action possible at that temperature. For comparative rates, therefore, we may take the *number of bacteria ingested per leucocyte per minute during the first half of the reaction; i.e.*, until one-half the maximum number of bacteria has been ingested. This criterion yields a value for the rate of the reaction which is far from being ideal but which seems to be the best approximation possible under the circumstances and certainly more rational than the original.

Following this procedure the rates of the reactions at different temperatures have been calculated from the data of Madsen and Watabiki.<sup>2</sup> In order to calculate from them the temperature coefficient,  $Q_{10}$ , of the reaction the logarithms of these rates have been plotted in Figs. 2 and 3 against the corresponding temperatures. The temperature coefficient for any interval of 10 degrees on the abscissæ is the antilog of the difference between the ordinates at the two temperatures; *i.e.*, the slope of the graph for that interval. The resulting graphs are practically straight lines which is rather an unusual result for biological processes. This means that the temperature coefficient is constant over the entire range from 5°–35°C.  $Q_{10}$  was found to be  $2.05 \pm 5$  per cent and  $2.0 \pm 5$  per cent in Figs. 2 and 3 respectively. For comparison with these curves the values of  $K$ , calculated by Madsen and Watabiki according to the formula for a monomolecular reaction, are also plotted in Figs. 2 and 3. Our improved analysis evidently gives a smoother curve, the probable error being only about one-half as large.

In a recent paper on phagocytosis (2) a formula was derived for calculating the chances of collision between leucocytes and particles of known size and density when stirred together in a common suspen-

<sup>2</sup> Madsen and Watabiki (1), Tables 1 and 2. The data on other tables could not be similarly treated because the experiments were not carried to completion.

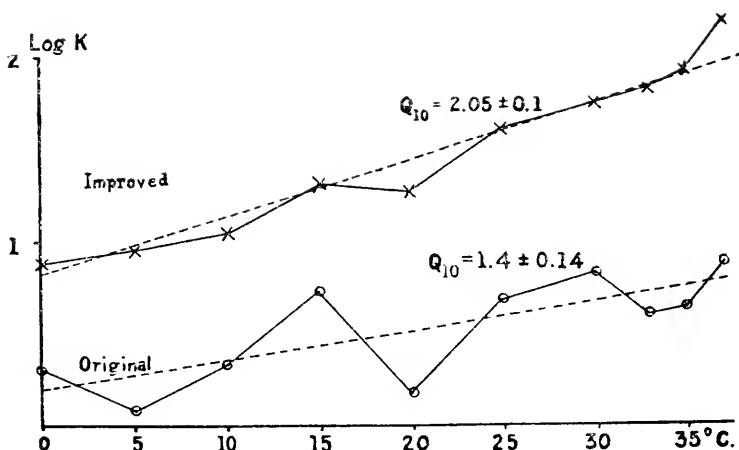


FIG. 2. Plots of the logarithms of  $K$ , the rate of phagocytosis (ordinates), against temperature (abscissæ). Data taken from Table 1 of Madsen and Watabiki. Lower curve from their own calculation of  $K$  by the formula of a monomolecular reaction. Upper curve shows the calculation of  $K$  by the improved method described in the text after correcting for viscosity of the medium. Slope of the graph (dotted line) represents the temperature coefficient of the process,  $Q_{10}$ , which is practically constant over this range of temperature. Calling the dotted line 100 per cent, the probable percentage deviation of the points from this line is only one-half as great by the new analysis. Before correcting for viscosity the new analysis gives higher values of  $Q_{10}$  than the original. Constants have been added to the values of  $\text{Log } K$  in both experiments for convenience in plotting.

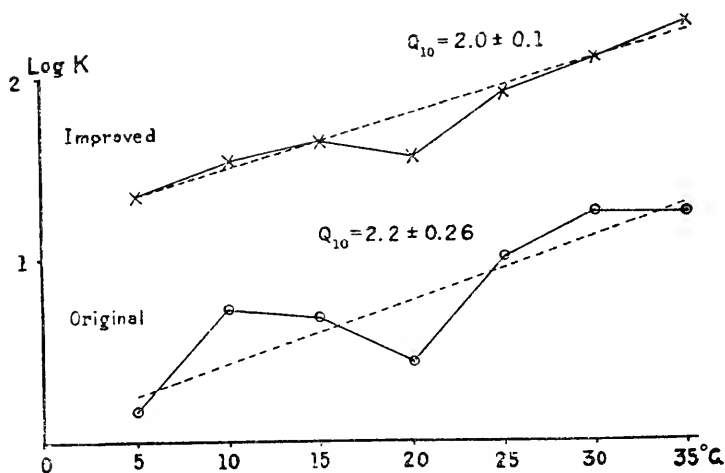


FIG. 3. Same as Fig. 1, but the data taken from another similar experiment of Madsen and Watabiki<sup>2</sup>.

sion. Since this formula was based on Stokes's law for falling bodies, the chances of collision are inversely proportional to the viscosity of the medium. In the previous experiments the viscosity could be neglected because it did not vary. Where temperature is the variable, however, it is obvious that the viscosity becomes a factor of some significance because it decreased proportionally the number of meetings between leucocytes and bacteria. The rates of phagocytosis calculated by the improved method were, therefore, corrected by multiplying by the viscosity of water as given in Landolt and Bornstein's tables before plotting. If the same correction had been applied to the original values of  $K$  they would have given a temperature coefficient less than the new values. The correction for viscosity decreases the value of  $Q_{10}$ , as shown in Table I, but does not appreciably affect the form of the curve.

It is conceivable that the number of collisions between bacteria and leucocytes might be so great that this would not be a limiting factor. In experiments on the phagocytosis of solid particles this certainly was not the case since the number of particles ingested in a given time was always a constant percentage of the number present.

The last column in Table I gives values for  $Q_{10}$  obtained when the time is measured from the end of the latent period instead of the beginning, the correction for viscosity also being made. This procedure gives a slightly lower value for  $Q_{10}$ .

We may take, then, for the temperature coefficient of phagocytosis, as nearly as it may be obtained from these experiments, the value 2.0.<sup>3</sup>

This figure is not thoroughly satisfactory, however, because the curves in Figs. 1 and 2 are complicated by at least two reactions, each with its own temperature coefficient, besides the phagocytic reaction proper.

The first of these reactions is represented by the latent period. Madsen and Watabiki calculated its temperature coefficient from the

<sup>3</sup> Ledingham, in studying the effect of temperature on phagocytosis of bacteria, concluded that it was due to the different rates of adsorption of opsonin by the bacteria. By previously incubating the bacteria with serum the accelerating effect of temperature could be nearly excluded. (Ledingham, J. C. G., *Proc. Roy. Soc. Biol., B.* 1907, lxxx, 188.)

length of the latent period by the formula of Arrhenius, and found  $\mu = 15,000$  and  $16,350$  respectively in their paper.<sup>2</sup> The data fit the simpler formula of van't Hoff equally well and gives  $Q_{10}$  as 2.35 and 2.6, respectively.

A second complicating reaction is evidenced in Fig. 1 by the fact that at higher temperatures phagocytosis ceases; *i.e.*, the maximum is reached sooner than at lower temperatures. This indicates that some secondary reaction is occurring which results in injury to the cells or otherwise makes phagocytosis impossible.<sup>4</sup> The speed of this "lethal reaction," as we may call it, increases with the temperature. If the phagocytic reaction itself were accelerated by rise of temperature

TABLE I.

*Temperature Coefficient of Phagocytosis.*

	Uncorrected.	Corrected for viscosity.	Latent period deducted.
	$Q_{10}$	$Q_{10}$	$Q_{10}$
Fig. 2.....	$2.7 \pm 0.1$	2.05	2.0
Fig. 3.....	$2.5 \pm 0.1$	2.0	1.9

to the same degree as the lethal reaction, the maximum reached would be the same at all temperatures, for the more rapid phagocytosis at higher temperatures would just compensate for the shorter time available. Therefore, the fact that the maximum attained is higher at the higher temperatures, in spite of the shorter time, proves that the temperature coefficient of phagocytosis must be higher than the temperature coefficient of the lethal reaction.

In Fig. 4 are plotted graphs from which the  $Q_{10}$  of the lethal reaction can be approximated. Ordinates represent logarithms of the reciprocals of the time necessary for completion of the reaction, *i.e.*, time to reach the maximum; abscissæ represent temperature. These figures are, of course, highly inaccurate, probably particularly so at tem-

<sup>4</sup> The maximum cannot be due entirely to any mechanical factor such as the exhaustion of the available bacteria or the filling up of the cells or it would be the same in all. The lethal reaction which determines the maximum might be referred to the toxic action of bacterial extracts.

peratures below  $15^{\circ}$  where the reactions were continued for 24 and 48 hours; and it has seemed justifiable, in calculating the value of  $Q_{10}$ , to discount these figures on account of secondary changes which might occur in such prolonged experiments. If this is done, the values for the temperature coefficient are 1.7 and 1.3 respectively. This bears out the qualitative conclusion arrived at from mere inspection of the curves in Fig. 1 that the temperature coefficient of the reaction resulting in injury to the cells was lower than that for the process of phagocytosis itself.

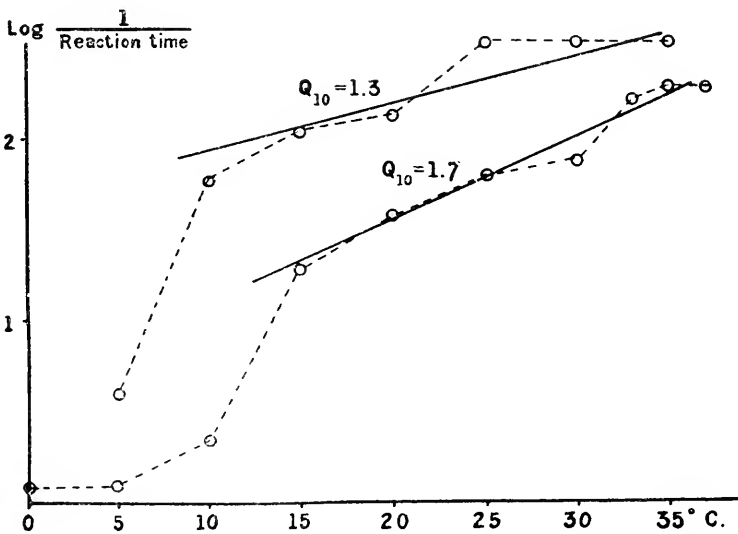
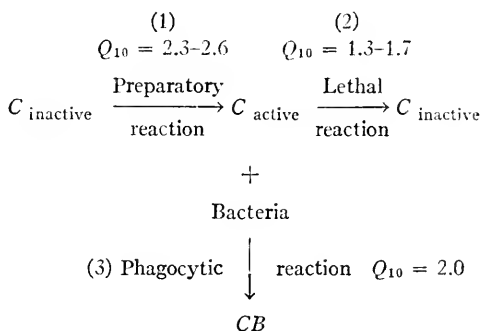


FIG. 4. Logarithms of the reciprocals of the times necessary to ingest the maximum number of bacteria (ordinates) plotted against temperature (abscissæ). Data taken from the same two experiments of Madsen and Watabiki as for the graphs of Figs. 2 (lower) and 3 (upper). Slope of the graph is proportional to the logarithm of the value of  $Q_{10}$  for that interval. Values calculated from the slopes of the solid lines are inserted. This is regarded as the temperature coefficient of the lethal reaction resulting in the death of the cell and cessation of phagocytosis. 3.75 and 2.5 have been added to the values for the upper and lower curves respectively for convenience in plotting.

Loeb (4) and Osterhout (5) have developed the conception of catenary reactions as applied to biological reactions, and the latter has recently discussed temperature coefficients from this point of view.

This general conception is obviously applicable to the experiments of Madsen and Watabiki, where three separate reactions can be clearly distinguished by mere inspection of their curves in Fig. 1. These reactions are: (1) a preparatory reaction represented by the latent period, during which it may be supposed that the leucocytes, originally inactive, are rendered active; (2) a lethal reaction which injures the leucocytes so that they are again inactive; and (3) the phagocytic reaction proper.

The relations between these three reactions may be diagrammed as follows:<sup>5</sup>



Here  $C$  stands for white blood corpuscles and  $CB$  for corpuscles containing bacteria. Now the rate of the phagocytic reaction (3) evidently depends upon the concentration of  $C_{\text{active}}$ ; and the accuracy of our analysis depends upon the assumption that this concentration is the same at different temperatures during the periods selected as corresponding periods in the reactions. The concentration of  $C_{\text{active}}$  depends in turn upon the relative rates of the preparatory and lethal reactions. Could we know the rates and dynamics of both these reactions as well as their temperature coefficients we could calculate the variation of  $C_{\text{active}}$  with time by Osterhout's equations (6) for the calculation of the concentration of  $M$  in the series  $A \rightarrow M \rightarrow B$ . In this way corresponding stages could be ac-

<sup>5</sup> In this diagram no assumption is implied as to whether the effect of reactions (1) and (2) in "activating" and "inactivating" the cells is due to an action upon the cells themselves or upon the medium or bacteria. It merely states the fact that their phagocytic activity passes through a maximum during the experiment, due to two reactions.



curately selected. Unfortunately this is not possible. Moreover, the activity of the cells cannot be quite the same during the periods selected, since the higher temperature coefficient of the preparatory reaction compared to the lethal reaction would make the concentration of  $C_{\text{active}}$  pass through its maximum sooner at higher temperatures. An improvement might be made by taking as corresponding stages the times until the lethal reaction is one-half complete; *i.e.*, one-half the time necessary for cessation of phagocytosis instead of the time for the ingestion of one-half the total number of bacteria. The validity of this method depends upon the doubtful assumption that cessation of phagocytosis is caused entirely by the lethal reaction. It seems quite probable, however, particularly at the higher temperatures, that the filling up of the leucocytes with bacteria or the partial exhaustion of the free bacteria is another factor of importance. There is obviously a limit to the accuracy of interpretation which is possible. On the whole, the corresponding stages in the reaction probably would not differ so much from those which we have used that the *average* rates of ingestion in those periods would be seriously affected.

In conclusion, emphasis may be laid upon the central fact that the phagocytic curves of Madsen and Watabiki represent a complex of at least three reactions and consequently cannot be treated as a single monomolecular reaction without serious error. Osterhout's conception of catenary and (we may add) collateral reactions is not only applicable to the interpretation of these experiments, but obviously essential.

For comparison with the results of Madsen and Watabiki on the temperature coefficient of the phagocytosis of bacteria, it seemed of interest to determine the temperature coefficient of the phagocytosis of solid particles of carbon and quartz. For this purpose leucocytes obtained from a peritoneal exudate in the rat and particles of quartz or carbon of uniform sizes (2 to  $4\mu$  in diameter) were mixed in small glass-stoppered vials which were rotated slowly about their horizontal axes in water baths kept at the desired temperature. At frequent intervals small samples were removed and counts made of the number of particles not yet ingested. For the details of the procedure the reader is referred to a previous paper (2). The difficulty with the method is that both particles and leucocytes are likely to agglutinate

more or less during the experiment and that it is impossible to be sure whether the particles are actually inside the cells or merely stuck on the outside. Hence, if the particles stick on the outside more rapidly than they are ingested, it becomes impossible to measure the actual rate of ingestion or to determine the true temperature coefficient of ingestion. This is undoubtedly the explanation of the low temperature coefficient, 1.4, obtained by this method between 30° and 37°C.

The results of one experiment by the suspension method is shown in Fig. 5, where the percentage of particles *not* ingested is plotted as ordinates against time in minutes as abscissæ. Determinations were made at 37°, 27°, and 23°C. Inspection of these curves shows that the temperature coefficient is very low above 30°C. compared to that below 30°C. To obtain a quantitative comparison, the times,  $T$ , necessary for ingestion of 25, 50, and 75 per cent of the particles have been compared. Here, again, the rate of the reaction is conditioned in part by the viscosity of the medium, collisions taking place between cells and particles with proportionally less frequency in the more viscous medium at 23°C. The rate of the reaction,  $K$ , when corrected for viscosity is, then, the reciprocal of the time necessary for the ingestion of a given percentage of particles multiplied by the viscosity.

$$K = \frac{V}{T} \quad (1)$$

The temperature coefficient,  $Q_{10}$ , is now calculated according to the formula

$$\text{Log } Q_{10} = \frac{\text{Log } K_1 - \text{Log } K_2}{t_1 - t_2} \times 10 \quad (2)$$

where  $t$  is the temperature, as already explained. In this case the graph of  $\log K$  against temperature is not a straight line as in the case of Madsen and Watabiki's results. In the experiment plotted in Fig. 5,  $Q_{10}$  is 1.26 between 23° and 27°C., and 5.6 between 27° and 37°C., when the figures for comparison are taken at the stage in the reaction when 50 per cent of the particles are ingested. The values obtained at other stages (25 and 75 per cent) in the reaction are given in Table II together with the results of seven other similar experiments. While the variations in the experimental figures are

large, the general tendency for  $Q_{10}$  to increase at lower temperatures is perfectly clean-cut.

The simplest interpretation of these results seems to be that at the lower temperatures the cells are actually too rigid to permit either the sticking on of the particles or the relatively greater changes in form which are necessary for ingestion. A slight increase in temperature now suffices to transform the protoplasm possibly from the *gel* to the

TABLE II.  
*Temperature Coefficient of Phagocytosis of Solid Particles.*

Experiment No.	Particles		25 per cent ingested.	50 per cent ingested.	75 per cent ingested.	Remarks.
	Nature.	Size.				
		$\mu$	$Q_{10}$	$Q_{10}$	$Q_{10}$	
1	Carbon.	4.7	1.1	1.3	1.0	Between 30°C. and 37°C.
2	Quartz.	6.0	1.9	1.6		
3	"	2.4	1.7	1.1	1.1	
4	Carbon.	4.7		1.2	1.4	Grand average = $1.41 \pm 0.06$ .
5	"	4.7	2.0	2.3	1.6	
6	"	4.7	1.4	1.3		
7	Quartz.	4.6	1.0	1.8	1.0	
8	"	2.4	1.3	1.2	1.3	
1	Carbon.	4.7	5.9	5.6	14.0	Between 23°C. and 27°C.
7	Quartz.	4.6	71.0	10.2	8.9	
9	Carbon.	4.7	10.7			Grand average = $18 \pm 6$ .

Comparisons were made at three different stages of the reaction; *i. e.*, when 25, 50, and 75 per cent of the particles had been ingested. In Experiments 1, 4, and 7,  $Q_{10}$  was measured from 27°C. to 37°C.

*sol* stage, thereby rapidly increasing the rate of phagocytosis; *i. e.*,  $Q_{10}$  is high. The low temperature coefficient above 30°C. might be taken to indicate that phagocytosis is merely a matter of surface tension changes. But this conclusion is not justified since many of the particles were merely stuck on the outside of the leucocytes singly or in clumps, making impossible any conclusion as to the temperature coefficient of the actual process of ingestion. The fact that no appreciable clumping occurs at lower temperatures shows that the

clumping is due to the increased stickiness or phagocytic activity of the cells.

In this connection the parallel fact is significant that the presence of solid particles has been found (2) to cause more rapid clumping of the leucocytes. Thus, at the optimum temperature for phagocytosis there is both an agglutination of cells due to particles and an agglutination of particles due to cells. The complete act of phagocytosis is

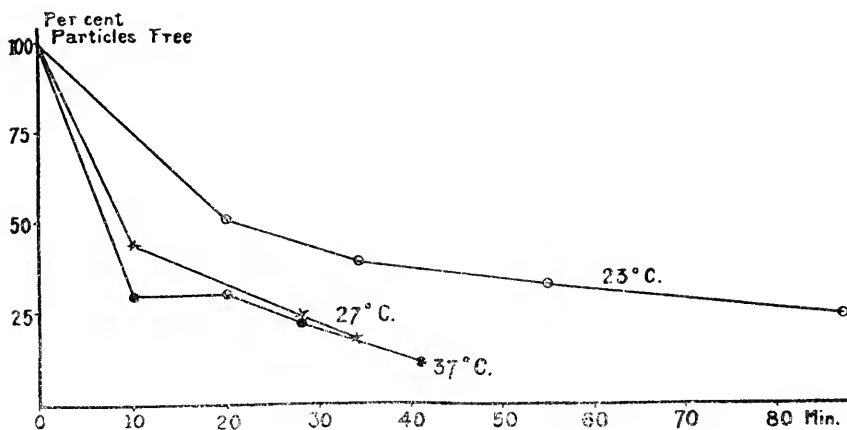


FIG. 5. Curves showing the number of particles of carbon still free (ordinates), *i.e.*, not ingested by leucocytes, as a function of time (abscissæ), at 23°, 27°, and 37°C. Inspection shows that the temperature coefficient is greater between 23° and 27°C. than between 27° and 37°C. See Table I for figures from this and other similar experiments.

evidently a complex of several reactions, and any figure which may be obtained for the temperature coefficient of the process must be interpreted from this point of view. The fact that  $Q_{10}$  is not constant must mean that new reactions become the limiting factors as the temperature changes. Below 30°C. the fluidity of the cell is the limiting factor. Above 30°C, it is the stickiness of the cells which would be expected to vary merely with the surface tension.

These results indicate then a marked change in the consistency of the protoplasm between 20° and 30°C. which did not appear in the

results of Madsen and Watabiki. Similar indications are found, however, in measurements of the temperature coefficient of the ameboid motion of leucocytes by Commandon (7) who took moving pictures of creeping leucocytes at 25°, 30°, and 35°C. and determined the rates of movement by subsequent measurements on the films. He gives the following figures:

Temperature.	Speed of leucocytes.	$Q_{10}$
°C.	$\mu$ per minute	
25	9.6	4.6
30	20.4	
30	20.4	1.5
35	25.2	

From these figures the  $Q_{10}$  has been calculated as described and was found to be 4.6 between 25° and 30°C., and 1.5 between 30° and 35°C.

Similar evidence of a rapid change in the temperature coefficient of ameboid movement below 30°C. was found in some preliminary experiments designed to measure the effect of temperature on phagocytosis by the "film method," previously described (2). In this method the leucocytes and particles are allowed to settle out between a slide and cover-slip in a thin film and the rate of phagocytosis is measured by counting the number of particles not yet ingested over equal areas. It is impossible to be sure, however, whether this rate is determined by the speed with which the leucocytes creep about from particle to particle or by the actual ease of ingestion. Demonstrations by this method, of the more rapid ingestion of carbon particles as compared with quartz, have shown that in this case at least the speed of the leucocytes is not the limiting factor. However that may be, the temperature coefficients of phagocytosis as measured by this method show the same marked increase at lower temperatures. This is shown in Table III.

TABLE III.

*Temperature Coefficients of Phagocytosis by Film Method.*

Experiment No.	Nature of particle	Particles ingested.	27°-35°C.	20°-27°C.	10°-20°C.
		<i>per cent</i>	$Q_{10}$	$Q_{10}$	$Q_{10}$
10	Carbon.	25	4.2	7.9	6.8
		50	4.2	10.0	6.0
		75	7.1		
	Quartz.	25	1.7	20.0	7.6
		50	1.3		
		75	2.0		
11	Carbon.	25	3.2	9.3	
		50	4.0	8.7	
	Quartz.	25	2.0	25.0	
		50	1.6	18.6	
Averages.....			3.2 ± 0.4	14.2 ± 1.7	6.8

In each experiment a mixture of quartz particles  $4.6 \mu$  in diameter and carbon particles  $4.7 \mu$  was used. 0.3 cc. of this mixture in distilled water was added to 1 cc. of leucocyte suspension plus 0.4 cc. serum plus 0.2 cc. NaCl 2.25 per cent plus 0.2 cc.  $\frac{M}{10}$  phosphate mixture of pH 7.5. Rates of phagocytosis,  $K$ , were taken equal to the reciprocal of the times,  $T$ , necessary for the ingestion of 25 per cent, 50 per cent, or 75 per cent of the particles.  $T$  was determined graphically.  $Q_{10}$  was calculated by equation (2). The dispersion of the average was calculated where the data were adequate.

## SUMMARY.

1. The experiments of Madsen and Watabiki on the effect of temperature on the phagocytosis of bacteria are discussed and a new analysis of their curves is given, showing that the rate of phagocytosis is very nearly a logarithmic function of the temperature from  $0^\circ$  to  $35^\circ\text{C}.$ ; *i.e.*,  $Q_{10}$  is constant over that range and is equal to 2.0.

2. New experiments are reported on the effect of temperature on the phagocytosis of quartz and carbon particles of uniform sizes, showing a marked increase in the temperature coefficient below  $30^\circ\text{C}.$

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## THE PENETRATION OF CATIONS INTO LIVING CELLS.

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According to some investigators the living cell is impermeable to salts, while according to others it is permeable to anions but not to cations. The object of the present investigation is to throw some light on the problem by making direct determinations of the penetrating substances.

The literature of the permeability of protoplasm to salts has been summarized by Brooks<sup>1</sup> and will not be discussed here. Attention may, however, be called to the fact that satisfactory direct methods of study have been lacking. The importance of obtaining direct evidence of the penetration of substances into the protoplasm cannot be overestimated.

The demonstration of direct penetration has been very difficult because individual cells are usually so small that their contents cannot be analyzed. Meyer,<sup>2</sup> Hansen,<sup>3</sup> Wodehouse,<sup>4</sup> and Crozier,<sup>5</sup> have examined the cell contents of *Valonia* for evidence of penetration of salts from sea water. In this case the cell sap can be obtained without contamination and in sufficient quantities for examination.

By employing a large form of *Nilella* the writer was able to investigate the penetration of several cations from balanced and from unbalanced solutions. This species of *Nilella* is especially favorable because of the length of the (multinucleate) cells (5 inches is not unusual), and the amount of cell sap which can be expressed from a single cell.

<sup>1</sup> Brooks, S. C., *Bot. Gaz.*, 1917, lxiv, 230.

<sup>2</sup> Meyer, A., *Ber. deutsch. bot. Ges.*, 1891, ix, 79.

<sup>3</sup> Hansen, A., *Mitt. Zool. Stat. Neapel*, 1893, xi, 255.

<sup>4</sup> Wodehouse, R. P., *J. Biol. Chem.*, 1917, xxix, 453.

<sup>5</sup> Crozier, W. J., *J. Gen. Physiol.*, 1918-19, i, 581.

The cells were placed in the solutions<sup>6</sup> for various lengths of time. When they were taken out they were thoroughly rinsed in running tap water, dried with filter paper and pierced with a fine pointed capillary pipette, which collected the cell sap in a very satisfactory manner. This was tested by means of the spectroscope.

The normal cell contained Na, Ca, and Cl; for this reason Na and Ca were not used in making up the test solutions.

The cations used were Li, Cs, and Sr. In order to avoid plasmolysis hypotonic solutions were employed.

In order to make sure that the tests obtained were not due to small quantities of the solution adhering to the outside of the cell, cells were placed for half an hour or longer in each solution, taken out, washed in running water, and tested in the usual manner. The test was in all cases negative, showing that the method as ordinarily used excludes errors due to contamination.

When *Nitella* was placed in 0.05 M LiCl, penetration of Li could be demonstrated in 24 hours. At this concentration the salt was not toxic. The cells remained in good condition for 4 days (after which the experiment was discontinued). Other plants which had been placed in 0.025 M LiCl gave a faint test for Li in 48 hours and were found to be in excellent condition at the end of 13 days.

In a balanced solution of Li (containing 10 parts 0.04 M LiCl, 9 parts tap water, and 1 part sea water) the cell sap gave a good test in 48 hours and cells were found to be in excellent condition at the end of 16 days.

Other cells were placed in 0.05 M CsCl and gave a good test in 24 hours. These were under observation for 6 days, during which time they remained in good condition.

In a balanced solution containing 10 parts 0.05 M CsCl, 9 parts tap water, and 1 part sea water, the cell sap gave a good test for Cs in 3 days, and the cells remained in good condition for 4 days (after which the experiment was discontinued).

In  $\text{SrCl}_2$  (0.075 M) *Nitella* remained alive for 20 days, and there was a slow penetration requiring 3 days or more to give a good test. The

<sup>6</sup> The solutions were approximately neutral. The temperature did not vary much from 19°C.

penetration of Sr from a balanced solution containing 10 parts  $\text{SrCl}_2$  (0.075 M), 9 parts tap water, and 1 part sea water, was still slower.

These experiments demonstrate that Li, Cs, and Sr penetrate into the interior of the living cell and that this occurs more rapidly in an unbalanced than in a balanced solution. That they may penetrate in the form of ions is evident from the fact that in salts of these metals the electrical conductivity<sup>7</sup> of the living cell is such as to show that the cations Li, Cs, and Sr readily penetrate the protoplasm. Since this occurs at the very start of the experiment, before any appreciable injury has occurred, it is evident that the cell in its normal condition is permeable to these cations.

#### SUMMARY.

Direct tests of the cell sap of *Nitella* show that the protoplasm is normally permeable to Li, Cs, and Sr, and that penetration is more rapid in an unbalanced than in a balanced solution.

<sup>7</sup> These results were obtained by the method described by Osterhout (Osterhout, W. J. V., *J. Gen. Physiol.*, 1921-22, iv, 275).



# THE ORIGIN OF THE ELECTRICAL CHARGES OF COLLOIDAL PARTICLES AND OF LIVING TISSUES.

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## *I. Stability of Suspensions, Electrical Charges of Micellæ, and Donnan Equilibrium.*

The stability of suspensions is, perhaps, the chief problem of a theory of colloidal behavior. Hardy has shown that this problem is linked with the problem of the origin of the electrical charges of the particles in suspension (which we will term micellæ, when they consist of aggregates of ions or molecules) inasmuch as the micellæ carrying a sufficiently large electrical charge will be forced by mutual electrostatic repulsion to stay in suspension. By his experiments on the migration of suspended particles of coagulated white of egg in an electrical field he proved that they have a positive charge in the presence of acid, a negative charge in the presence of alkali, and no charge at an intermediate point which he termed the isoelectric point of the particles. He was able to demonstrate that the stability of colloidal suspensions is a minimum at the isoelectric point.<sup>1</sup>

He and others found, moreover, that low concentrations of neutral salts diminish the stability of colloidal suspensions in the presence of acids or alkalies and that the efficient ion of the salt has the opposite sign of charge from the colloidal particle; since the precipitating efficiency of a salt increases rapidly with the valency of that ion of the salt which has the opposite sign of charge from the colloidal particle. It seemed natural to infer that the precipitation of colloidal

<sup>1</sup> Hardy, W. B., *Proc. Roy. Soc. London, Series B*, 1899-1900, lxvi, 110; *J. Physiol.*, 1903, xxix, p. xxvi. Wood, T. B., and Hardy, W. B., *Proc. Roy. Soc. London*, 1909, lxxxi, 38.

suspensions by low concentrations of a salt was caused by an annihilation of the charge of the colloidal particle. The problem of the stability of the colloidal suspension then developed into the problem of accounting for this peculiar behavior of the electrical charges of colloidal particles.

Hardy's original idea was that the H ions of the acid or OH ions of the alkali were adsorbed by the colloidal particle in preference to the other ions on account of their greater rapidity of migration; and this idea was also accepted by Perrin in his experiments on electrical endosmose, where it was necessary to account for the fact that certain membranes become positively charged in the presence of acid and negatively in the presence of alkali.<sup>2</sup> Those who accept this adsorption hypothesis explain the fact that the electrical charges of the particles are apparently diminished or destroyed by the addition of a salt on the assumption of a preferential adsorption of one of the ions of the salt; yet such an assumption is incompatible with the purely stoichiometrical behavior of proteins. It is also difficult to account for the fact that the addition of little acid increases while the addition of more acid depresses the electrical charge of micellæ on the basis of the adsorption hypothesis.

A second possibility was pointed out by the writer in 1904; namely, that Hardy's migration experiments might be explained in the case of proteins by the fact that proteins are amphoteric electrolytes which, in the presence of alkali, dissociate electrolytically by giving rise to a protein anion and, in the presence of acid, by giving rise to a protein cation while at the isoelectric point no protein ion would be formed.<sup>3</sup> While this idea is correct if applied to the migration of isolated protein ions in the electrical field, it cannot explain why the addition of a salt in low concentration should diminish the charge of aggregates of molecules and ions, the micellæ, except by assuming that in this case the electrolytic dissociation of the protein salts should be repressed. The concentration of salts required for the precipitation of colloidal suspensions is, however, much too small to make such a suggestion acceptable.

<sup>2</sup> Perrin, J., *J. chim. physique*, 1904, ii, 601; 1905, iii, 50.

<sup>3</sup> Loeb, J., *Univ. California Pub., Physiol.*, 1903-04, i, 149.

In 1916 J. A. Wilson suggested that the electrical charges of micellæ were caused by the establishment of a Donnan equilibrium between the colloidal particle and the surrounding solution.<sup>4</sup> There were, however, no measurements of membrane potentials available at that time and this was probably the reason that his suggestion was not accepted.

We may consider a solution of a protein inside a collodion bag surrounded by a watery solution (free from protein) as a model of a micella. The measurements of the p. d. with the aid of a Compton electrometer with saturated calomel electrodes show that the electrical charges of this model vary in the same way as the charges possessed by colloidal particles in general (*e.g.* coagulated egg albumin) in suspension; namely, (1) The electrical charge of the micella model is zero at the isoelectric point of the protein. (2) The charge of the model is positive on the acid side and negative on the alkali side of the isoelectric point, and increases with the addition of little acid and diminishes with the addition of more acid to isoelectric particles. (3) The charge of the model is depressed by the addition of low concentrations of neutral salts and the depressing action of the salt increases rapidly with the valency of that ion of the neutral salt which has the opposite sign of charge to that of the micella.<sup>5</sup>

If these charges are due to the Donnan equilibrium it must be possible to prove that the concentration of the crystalloidal ions inside the micella (or its model) is different from their concentration in the surrounding liquid and that this difference in the concentration of crystalloidal ions on the opposite sides of the membrane is able to account quantitatively for the observed p. d. The difference in the concentration of crystalloidal ions in the two phases (micella and surrounding water) is due to the fact that the protein ion cannot diffuse into the watery solution. When a solution of a protein-acid salt is inside a collodion membrane, the diffusion of the protein ion is prevented by the membrane and when the protein ion forms part of a gel the diffusion of the protein ions is prevented by the forces of

<sup>4</sup> Wilson, J. A., *J. Am. Chem. Soc.*, 1916, xxxviii, 1982.

<sup>5</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 667.

cohesion between the protein ions constituting the gel, while both the collodion membrane and the solid gel are permeable for crystalloidal ions. We have already shown that when we separate a solution of gelatin chloride from water by a collodion membrane, at equilibrium the concentration of chlorine ions inside the gelatin solution is greater than the concentration of chlorine ions in the outside solution, and the concentration of the hydrogen ions inside the gelatin solution is smaller than outside, as Donnan's theory demands.<sup>5</sup> The concentration of the Cl ions was determined by titration and it was shown that the P.D. calculated with the aid of Nernst's formula from the difference of concentration of chlorine ions inside and outside, was within the limits of accuracy of the measurements, identical with the P.D. directly observed between the gelatin chloride solution and the outside solution with the aid of a Compton electrometer with saturated calomel electrodes. In other words, it was found that

$$\text{P.D. observed} = 58 \log \frac{C_{\text{Cl outside}}}{C_{\text{Cl inside}}} \text{ millivolts}$$

or, since  $\log C_{\text{Cl outside}} = \text{pCl outside}$ , and  $\log C_{\text{Cl inside}} = \text{pCl inside}$ ,

$$\text{P.D. observed} = 58 \cdot (\text{pCl outside minus pCl inside}) \text{ millivolts}$$

If Donnan's theory accounts quantitatively for the observed P. D., the value 58 (pH inside minus pH outside) should also agree quantitatively with the observed P.D.

The values of pH inside and pH outside were determined in our experiments with the potentiometer (*i.e.* the hydrogen electrode), and the value 58 (pH inside minus pH outside) was therefore also an observed value. In order to avoid confusion of terms we call the P.D. observed with the Compton electrometer the *observed* P.D., since this gives us the empirical charge found in the micella or its model, free from any theory. On the other hand, we will call the value 58 (pH inside minus pH outside) the *calculated* P.D. since we could find this value by calculation from Nernst's formula if we determined the hydrogen ion concentration of the inside and outside solutions by titration instead of by the potentiometer.

The determinations of the value pH inside minus pH outside give the following results already published in a preceding paper:<sup>5</sup> (1)



At the isoelectric point of gelatin the value of pH inside minus pH outside is zero. (2) When the collodion bag contains a solution of gelatin-acid salt, the value pH inside minus pH outside is positive. (3) The value pH inside minus pH outside increases at first when acid is added to isoelectric gelatin with the increase in acid, but soon reaches a maximum and diminishes again upon the addition of further acid. It is shown that the p.d. observed with the Compton electrometer between the solution and the water varies in exactly the same way. (4) The addition of a neutral salt to a solution of gelatin chloride at the pH where the observed p.d. is about a maximum diminishes the value of pH inside minus pH outside in the same way as it diminishes the observed p.d. (5) The main fact was that the value 58 (pH inside minus pH outside) agreed quantitatively with the observed p.d.

These facts show that the p.d. between a gelatin chloride solution and a watery solution (separated by a collodion membrane) is caused exclusively by a difference in the concentration of diffusible ions inside and outside the gelatin solution. If there were a second source for the p.d., the p.d. obtained from the value 58 (pH inside minus pH outside) would be always smaller than the p.d. observed with the aid of a Compton electrometer. The reader will therefore see that the quantitative agreement between the values of 58 (pH inside minus pH outside) with the observed p.d. between the gelatin chloride solution and the outside solution is the essential proof that only the Donnan equilibrium is responsible for the difference of potential between the gelatin chloride solution and an outside watery solution.

This paper intends to fill out several gaps left in the preceding publication. Thus it was not proven that on the basis of the Donnan equilibrium the gelatin must have a negative charge on the alkali side of the isoelectric point. When we put a solution of Na gelatinate into a collodion bag and dip the bag into water, the Donnan equilibrium demands that NaOH be expelled from the solution of Na gelatinate through the collodion membrane into the outside solution, and that when equilibrium is established between the solutions of Na gelatinate and water the concentration of NaOH must be greater in the outside watery solution than in the solution of Na

gelatinate (inside solution). As a consequence the pH in the outside solution should be higher than in the inside solution, and the value pH inside minus pH outside should become negative when the inside solution is Na gelatinate. This is the reason why powdered particles of Na gelatinate must assume a negative charge. In the case of a gelatin chloride solution the value pH inside minus pH outside is positive and this explains why powdered particles of gelatin chloride are positively charged.

We will now show that when we separate a solution of Na gelatinate from a watery solution by a collodion membrane and allow both solutions to reach or approach equilibrium, the value pH inside minus pH outside actually becomes negative.

## *II. The Electrical Charge of Na Gelatinate.*

It is necessary to prevent the  $\text{CO}_2$  of the air from diffusing into the solutions of Na gelatinate, and therefore the outside solution was put into stoppered bottles connected with the outside air by glass tubes filled with soda lime. The pH measurements were less certain than in the experiments with acid on account of the  $\text{CO}_2$  error. There may be other experimental shortcomings, *e.g.*, the slowness of the establishment of the Donnan equilibrium between inside and outside solutions near the isoelectric point.

Collodion bags of a volume of about 50 cc. were filled with solutions of Na gelatinate containing 1 gm. of originally isoelectric gelatin and varying amounts of 0.1 N NaOH in 100 cc. solution. The collodion bags were dipped into flasks containing 500 cc. of aqueous solutions of NaOH of various concentrations and free from gelatin. The flasks were sealed, communicating with the air only through tubes filled with soda lime, as stated. The collodion bags containing the gelatin were closed by a rubber stopper perforated by a glass tube which served as a manometer. The experiment lasted 6 hours at a temperature of  $24^\circ\text{C}$ . The results of the experiments are given in Table I. The upper horizontal row gives the number of cc. of 0.1 N NaOH originally in 100 cc. of the gelatin solution; the second row gives the original concentration of NaOH in the outside aqueous solution free from gelatin; the third row gives the osmotic pressure in millimeters  $\text{H}_2\text{O}$  after 6 hours. The next row gives the pH inside

TABLE I.

*1 Per Cent Na Gelatinate.*

	0	1	2	3	4	5	6	8	10	12.5	15	20
	0	0	0	N/25,600	N/12,800	N/6,400	N/3,200	N/1,600	N/800	N/400	N/200	N/100
Cc. 0.1 N NaOH added to 1 gm. gelatin in 100 cc.....												
Concentration of NaOH of outside solution.....												
Osmotic pressure, millimeters.....	26	164	265	353	375	385	366	335	340	265	192	150
pH inside.....	5.02	5.40	5.76	6.64	7.15	9.02	9.68	10.16	10.45	—	11.30	11.58
pH outside.....	5.60	5.82	5.92	6.37	7.70	9.50	9.96	10.60	10.85	—	11.46	11.70
pH inside minus pH outside.....	-0.58	-0.42	-0.16	+0.27	-0.55	-0.48	-0.28	-0.44	-0.40	—	-0.16	-0.12
P.D. calculated, millivolts.....	-34.0	-24.5	-9.4	+15.8	-32.0	-28.0	-16.5	-25.7	-23.4	—	-9.4	-7.0
P.D. observed, millivolts.....	-3.5	-19.5	-18.0	-37.5	-37.5	-36.0	-30.0	-22.0	-19.5	—	-10.0	-7.0

and the following row the pH outside after the experiment was finished (*i.e.* after 20 hours), and the sixth row gives the difference pH inside minus pH outside. The reader will notice that this difference is always negative with one exception which is obviously an error. The last two rows give the calculated P.D. (*i.e.* the value 58 (pH inside minus pH outside)), and the observed P.D. Observed P.D. means the millivolts between the solution of Na gelatinate and watery solution measured directly in the Compton electrometer, as described in preceding papers.

It is obvious that there is no quantitative agreement between the observed and calculated P.D. near the isoelectric point, probably on account of the CO<sub>2</sub> error. As soon as the pH is above 7.0 the agreement between observed and calculated P.D. becomes better so that we are entitled to say that the difference of potential between a Na gelatinate solution and an outside solution is due to the Donnan equilibrium which forces the expulsion of NaOH from the inside into the outside solution. As a consequence the pH inside becomes lower than the pH outside.

### *III. Valency Effect on the P.D.*

It had been shown in a preceding paper that the charge of a solution of gelatin sulfate is lower than a charge of a solution of gelatin chloride or gelatin phosphate or any other gelatin-acid salt with a monovalent anion of the same pH and the same concentration of originally isoelectric gelatin.<sup>5</sup> It should, however, be pointed out that on the basis of the theory the ratio of the charges in the two cases should be exactly as 3:2. It is needless to say that if we can prove that this postulate is fulfilled the probability that the charges of micellæ are due to the Donnan equilibrium becomes very strong.

The equilibrium equation which is of the second degree when the anion is monovalent, *e.g.* Cl, in the case of gelatin chloride, becomes of the third degree when the anion is bivalent, *e.g.* SO<sub>4</sub>, in the case of gelatin sulfate. Let  $x$  be the concentration of hydrogen ions and Cl ions of the outside,  $y$  that of the H and Cl ions of the free HCl inside the gelatin chloride solution, and  $z$  the concentration of the Cl in combination with gelatin. Then the equilibrium equation is,

$$x^2 = y(y+z)$$

$$x = \sqrt{y(y+z)}$$

Substituting this term  $\sqrt{y(y+z)}$  for  $x$  in  $\frac{x}{y}$  we get

$$\frac{x}{y} = \frac{\sqrt{y(y+z)}}{y} = \sqrt{1 + \frac{z}{y}}$$

The p.d. is  $58 \log \sqrt{1 + \frac{z}{y}} = \frac{58}{2} \log \left(1 + \frac{z}{y}\right)$ .

In the same way we can arrive at the term for  $\frac{x}{y}$  in the case of gelatin sulfate solution. If we call  $x$  the concentration of hydrogen ions in the outside solution,  $y$  that of the hydrogen ion concentration in the inside solution; then  $\frac{x}{2}$  is the concentration of the  $\text{SO}_4$  ions in the outside and  $\frac{y}{2}$  the concentration of the  $\text{SO}_4$  ions of the free  $\text{H}_2\text{SO}_4$  in the inside (gelatin) solution. The concentration of  $\text{SO}_4$  ions in combination with gelatin becomes  $\frac{z}{2}$ . Then the equilibrium equation is as follows:

$$\frac{x^3}{2} = \frac{y^2(y+z)}{2}; \quad x = [y^2(y+z)]^{\frac{1}{3}}$$

The value which interests us is  $\frac{x}{y}$ , i.e. the ratio of the hydrogen ion concentration outside over that inside.

Substituting  $[y^2(y+z)]^{\frac{1}{3}}$  for  $x$  in  $\frac{x}{y}$  we get

$$\frac{x}{y} = \sqrt[3]{\frac{y^2(y+z)}{y}} = \sqrt[3]{\frac{y+z}{y}}$$

The p. d. is therefore in the case of gelatin sulfate

$$\text{p.d.} = \frac{58}{3} \log \left(1 + \frac{z}{y}\right) \text{ millivolts}$$

while in the case of gelatin chloride it is

$$\text{p.d.} = \frac{58}{2} \log \left(1 + \frac{x}{y}\right) \text{ millivolts}$$

Hence the P.D. of gelatin sulfate solution should be two-thirds of the P. D. of a gelatin chloride solution of the same pH and the same concentration of originally isoelectric gelatin.

1 gm. of isoelectric gelatin was dissolved in 100 cc. of water containing in one case 5 cc. of 0.1 N HCl, in the other, 5 cc. of 0.1 N H<sub>2</sub>SO<sub>4</sub>. One collodion bag with a volume of 50 cc. was filled with the gelatin chloride solution and this bag was dipped into a beaker containing 350 cc. N/1000 HCl. A second collodion bag was filled with the gelatin sulfate solution and this bag was dipped into 350 cc. N/1000 H<sub>2</sub>SO<sub>4</sub>. The solutions were kept for 24 hours at 24°C. and the pH inside, and pH outside were measured. The pH of the two gelatin solutions was identical, namely 3.64, but the value pH inside minus pH outside was for the gelatin chloride solution 0.49 and for the gelatin sulfate solution 0.31, which is as near 3:2 as the accuracy of the measurements permits. A confirmation of this result can be found in the experiments published in a preceding paper where this relation had not yet been recognized.<sup>5</sup> Thus it was found that for gelatin phosphate of pH 3.98 the value of pH inside minus pH outside was 0.58, while for gelatin sulfate of pH 3.98 the value of pH inside minus pH outside was 0.38, which is again the ratio of 3:2. For pH 4.31 the value pH inside minus pH outside was 0.53 for gelatin chloride while it was for pH 4.34, 0.35 for gelatin sulfate, which is again as 3:2. The P.D. observed directly with the Compton electrometer agreed quantitatively with the value 58 (pH inside minus pH outside).

Quantitative results, such as these, leave little doubt that the P. D. between solutions of gelatin-acid salts and outside watery solutions when separated by a collodion membrane are determined entirely by the Donnan equilibrium; and that there can be no other source of the charge of this micella model.

#### *IV. The P. D. of Solutions of Crystalline Egg Albumin.*

The experiments published thus far had all been done on gelatin. It was of importance to make sure whether or not these results can be confirmed with crystalline egg albumin. This was found to be the case, and the experiments on the membrane potentials of the

solutions of the chloride of crystalline egg albumin showed a perfect quantitative agreement with the theory.

Collodion bags of about 50 cc. volume were filled with a solution of 1 per cent crystalline egg albumin containing varying amounts of 0.1 *N* HCl, and the bags were put, as usual, into beakers containing 350 cc. of HCl solutions of different concentration but free from albumin. The first two horizontal rows of Table II give the amount of 0.1 *N* HCl in each solution. The experiments were carried out at a temperature of 24°C. and after 22 hours the osmotic pressure, *P. D.*, and pH of inside (albumin) solution and pH of the outside solution were measured, the *P. D.* with the Compton electrometer and the pH with the hydrogen electrode. The albumin used was not isoelectric, but, since it had been prepared after Sørensen's method, it was probably partly ammonium albuminate, with a pH of near 6.0. The table shows that the observed *P. D.* agree with the value 58 (pH inside minus pH outside), *i.e.* the calculated *P. D.* (especially on the acid side of the isoelectric point); that the *P. D.* is a minimum near pH 4.7 of the albumin (*i.e.* near its isoelectric point which is at pH 4.8), and that the albumin is positively charged on the acid and negatively charged on the alkaline side of the isoelectric point. This is again in harmony with what we should expect on the basis of the Donnan equilibrium.

The next problem was to determine the influence of the addition of a neutral salt to a solution of the chloride of crystalline egg albumin. A 1 per cent solution of crystalline egg albumin containing 7 cc. of 0.1 *N* HCl in 100 cc. was made up in various concentrations of NaCl. The collodion bags containing these albumin chloride-NaCl mixtures were dipped into beakers containing 350 cc. of the same concentration of NaCl as that of the albumin solution, and all made up in *N*/1000 HCl. The experiment was carried out at 24°C. and the measurements were made after 22 hours.

Table III gives the results which show again a good agreement between the observed *P. D.* and the value 58 (pH inside minus pH outside), our so called calculated *P.D.*

We may, therefore, conclude that the *P.D.* of both gelatin solutions and solutions of crystalline egg albumin separated by a collodion membrane from a watery solution free from protein is accounted for

TABLE II.  
*1 Per Cent Albumin Chloride.*

Cc. 0.1 N HCl added to 1 gm. albumin in 100 cc. ....	0	1	2	3	4	5	6	7	8	10	15	20	30	40
Cc. 0.1 N HCl in 350 cc. outside solution .....	0	0.1	0.3	0.5	1	1.5	2.1	2.8	4	7.1	16.4	32	60	80
Osmotic pressure, millimeters.....	155	100	52	114	178	205	214	219	218	180	136	100	81	74
pH inside.....	5.80	5.40	4.70	4.30	4.00	3.75	3.64	3.42	3.24	3.00	2.53	2.20	1.89	1.73
pH outside.....	6.14	5.64	4.67	4.06	3.65	3.38	3.22	3.07	2.91	2.71	2.37	2.10	1.82	1.70
pH inside minus pH outside.....	-0.34	-0.24	+0.03	+0.24	+0.35	+0.37	+0.42	+0.35	+0.33	+0.29	+0.16	+0.10	+0.07	+0.03
p.d. calculated, mil- livolts.....	-20.0	-14.0	+2.0	+14.0	+20.6	+22.4	+25.5	+21.0	+20.0	+17.5	+9.4	+6.0	+4.0	+2.0
p.d. observed, mil- livolts.....	-24.0	-16.0	+3.0	+11.5	+19.0	+19.5	+20.5	+19.5	+18.5	+16.0	+11.0	+10.0	+4.0	+3.5



TABLE III.  
*Influence of Salt on p.D. of Albumin Chloride Solution.*

Concentration of NaCl.										
	0	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
Osmotic pressure, millimeters.....	210	181	156	131	107	87	73	61	54	45
pH inside.....	3.35	3.32	3.32	3.27	3.25	3.20	3.19	3.22	3.21	3.22
pH outside.....	3.04	3.04	3.07	3.10	3.11	3.13	3.14	3.18	3.21	3.23
pH inside minus pH outside.....	0.31	0.28	0.25	0.17	0.14	0.07	0.05	0.04	0.00	-0.01
p.D. calculated, millivolts.....	+18.0	+16.2	+14.5	+10.0	+8.0	+4.1	+2.9	+2.3	0	-0.5
p.D. observed, millivolts.....	+18.5	+15.5	+13.5	+10.0	+7.5	+5.0	+3.0	+1.5	+1.0	+0.5

completely by the Donnan equilibrium. There can be no other source for the electrical charge of this model of a protein micella except that due to the membrane equilibrium.

*V. The Electrical Charges of Suspended Particles of Powdered Gelatin.*

It is possible to show that the electrical charges of the powdered particles of gelatin suspended in a watery solution are determined by the fact that acid is forced from the suspended particles into the watery solution when the particles consist of gelatin chloride, and that alkali is forced from the particles into the solution when they consist of Na gelatinate.

Measurements of the p.d. between solid gels of gelatin and the surrounding solution suffer from inaccuracies (especially near the isoelectric point) which we have not been able to eliminate, so that we must be satisfied with only an approximate confirmation of the theory. In order to prove or to make it probable that the p.d. is due to the Donnan equilibrium we must be able to show that there exists a difference of the value of pH inside and pH outside the gel when the suspended particles of gelatin chloride or Na gelatinate are in equilibrium with the watery solution.

1 gm. of powdered gelatin of grain size between mesh 30 and 60, and rendered isoelectric was put into each of a series of closed flasks containing 350 cc. of distilled water with varying quantities of 0.1 N HCl or NaOH per 100 cc. (see Table IV). The temperature was 20°C. After 4 hours the powdered gelatin was separated from its liquid by filtration, the gelatin was melted and the pH of the melted gelatin and of the outside solution (filtrate) were measured. The gelatin was then solidified and the p. d. between the solid gelatin and the filtrate (outside solution) determined, as will be described a little later. The results of the experiments are given in Table IV. The first row gives the number of cc. of 0.1 N HCl or NaOH contained originally in 100 cc. outside solution. The next row gives the relative volume of the solid mass of gelatin, *i.e.* the degree of swelling. The rest of the table needs no explanation. It is obvious that pH inside minus pH outside is positive as long as the pH of the gelatin is on the acid side of the isoelectric point, while it is negative when the gelatin is on the alkaline side of the isoelectric point. The turning point is

TABLE IV.  
*Suspensions of Powdered Gelatin.*

	HCl.					NaOH.						
	1.0	0.5	0.2	0.1	0	0.1	0.2	0.5	1.0	2.0	4.0	
Cc. 0.1 N HCl or NaOH in 100 cc. solution.....												
Volume of gelatin, millimeters.....	28	20	18	16	17	18	28	37	40	47	48	
pH of melted gelatin (inside).....	4.44	4.56	4.79	4.85	4.89	4.98	5.06	5.50	6.74	9.54	10.15	
pH of supernatant liquid (outside).....	3.35	3.55	3.92	4.24	4.97	5.96	6.24	6.46	7.30	10.56	11.08	
pH inside minus pH outside.....	+1.09	+1.01	+0.87	+0.61	-0.08	-0.98	-1.18	-0.96	-0.56	-1.02	-0.93	
p.D. calculated, millivolts.....	+63.0	+58.6	+51.0	+36.0	-4.5	-57.0	-68.0	-56.0	-33.0	-59.0	-48.0	
p.D. observed, millivolts.....	+56.0	+55.5	+36.5	+15.0	-17.5	-59.0	-61.0	-70.0	-66.0	-46.0	-36.0	

approximately at the isoelectric point, but the measurements near the isoelectric point are obviously vitiated by experimental errors or by some other factor so that we cannot demonstrate more by the experiment than that the suspended particles of solid metal gelatin have the opposite sign of charge from the gelatin chloride and that this difference is accompanied by a reversal of the sign of the value of pH inside minus pH outside, which is positive in the case of gelatin chloride and negative in the case of Na gelatin. It may also be pointed out that the minimum of swelling (volume) coincides with the minimum of P.D.

While the experimental errors are rather great in the neighborhood of the isoelectric point and on the alkaline side, they are fortunately less annoying on the acid side when the hydrogen ion concentration is sufficiently large. In this case the agreement between the value pH inside minus pH outside and the P.D. observed is at least sufficient to show that the P.D. is caused by the Donnan equilibrium.

1 gm. of powdered isoelectric gelatin going through mesh 30 but not through mesh 60 was put into 350 cc. of water containing various quantities of HCl (see first horizontal row of Table V), and left in this solution for 24 hours at 20°C. The flasks were occasionally stirred. After 24 hours the volume of the particles was measured and they were put on a filter to allow the outside solution to drain off. The gelatin was then melted by heating to 45°C. and poured into glass cylinders which at their lower end had two glass side tubes attached. The mass was then allowed to solidify and the P. D. between gelatin and watery solution was ascertained. One of the two glass tubes dipped into a beaker containing the outside HCl solution (the filtrate) with which the gelatin had been in equilibrium, and the other dipped into a beaker containing a saturated solution of KCl. Each beaker was connected with one of the calomel electrodes (filled with saturated KCl) of a Compton electrometer. The last row in Table V gives the observed P.D. in millivolts.

The gelatin was then melted and its pH was determined potentiometrically. This is called pH inside in Table V. The pH of the outside solutions (filtrate) was also determined at 24°C.

While the agreement between the observed P.D. and the values of 58 (pH inside minus pH outside) is not as complete as in the experi-

TABLE V.  
*Suspensions of Powdered Gelatin.*

Cc. 0.1 N HCl in 100 cc. H <sub>2</sub> O. . . .	0.5	1.0	2.0	4.0	6.0	8.0	10.0	12.0	15.0	20.0	30.0	40.0
Volume of gelatin, millimeters. . .	30	40	62	73	75	73	66	64	54	50	41	37
pH of melted gelatin (inside) . . . .	4.58	4.27	3.76	3.26	2.92	2.57	2.41	2.29	2.11	1.96	1.78	1.59
pH of supernatant liquid (outside)	3.89	3.45	3.04	2.65	2.44	2.27	2.16	2.07	1.95	1.82	1.65	1.49
pH inside minus pH outside. . . . .	0.69	0.82	0.72	0.61	0.48	0.30	0.25	0.22	0.16	0.14	0.13	0.10
p.D. calculated, millivolts. . . . .	+40.7	+48.4	+42.5	+36.0	+28.4	+17.7	+14.7	+13.0	+9.5	+8.3	+7.7	+5.9
p.D. observed, millivolts. . . . .	+37.5	+39.0	+38.0	+29.5	+22.0	+17.7	+17.7	+18.2	+17.0	+10.7	+8.6	+5.4

ments with solutions inside collodion bags, it is at least sufficient to leave no doubt that this difference in pH inside and outside causes the P.D. In other words, there is no doubt that the P.D. between the powdered particles and the surrounding liquid with which they are in equilibrium is due to the Donnan equilibrium.

We have already shown in a preceding paper that the addition of a salt to a solution containing suspended particles of powdered gel of gelatin chloride diminishes the P.D. between the particles and surrounding liquid and that this diminution is due to a diminution of the value pH inside minus pH outside; *i.e.*, to the Donnan equilibrium.<sup>5</sup>

These facts then leave no doubt that the difference in the hydrogen ion concentration between micellæ of protein and the surrounding solution which the Donnan equilibrium demands is the only cause of the electrical charges of micellæ of proteins or of their models.

The experiments on the solution of casein chloride published by Robert F. Loeb and the writer in the preceding number of this Journal indicate that aside from the electrical charges osmotic forces may play a rôle in maintaining the stability of colloidal suspensions.<sup>6</sup> These forces are also a consequence of the Donnan equilibrium and hence vary in a similar way as the P.D. No other theory except the Donnan theory can account for this similarity.

#### *VI. The Origin of the Electrical Charges of Living Cells and Tissues.*

In his first paper on the theory of membrane equilibria Donnan suggested that the membrane potentials postulated by his theory might contribute towards an explanation of the action of nerves and even of electrical fish. In 1911 the writer suggested to Dr. Beutner that he investigate the P.D. between such organs as apples or leaves of the rubber plant and water, instead of the P.D. of muscles or nerves which had usually been used by physiologists for this purpose. In these experiments Dr. Beutner made the important observation that the P.D. between the surface of an apple or a leaf was a maximum when the bounding liquid was pure water, while the P. D. was depressed when a salt was added to the water the depressing effect on

<sup>6</sup> Loeb, J., and Loeb, R. F., *J. Gen. Physiol.*, 1921-22, iv, 187.

the P. D. increasing with the concentration of the salt.<sup>7</sup> MacDonald<sup>8</sup> had observed a similar phenomenon; namely, the increase in P.D. between nerve and surrounding salt solution with increasing dilution. Donnan's theory was not known to us and we were not able to give an explanation of the depressing effect of salt on the P.D.

We next searched for those substances in the cortex of an apple or leaf which might be responsible for these peculiar concentration effects on the P. D. When the P. D. between solid gels of gelatin and of coagulated egg albumin and water was investigated no potential differences were observed,<sup>9</sup> to the great surprise and disappointment of the writer who had hoped that the investigations of the P. D. might lead to an explanation of the antagonistic ion effects in which he was then interested. It is possible that the negative results with protein were due to the fact that the measurements were accidentally made near the isoelectric point. On the other hand, it was found that there exists a P.D. at the boundary of lipoids (lecithin dissolved in guaiacol) which is depressed by the addition of salts, and the more the higher the concentration of the salt.<sup>9</sup>

This analogy between lipoids and living cells gave us the impression that the proteins had no share in the potential differences observed between living tissues or living cells and watery solutions. The experiments recorded in this paper leave no doubt that this conclusion was wrong; any ion in a cell or on its surface which cannot diffuse into the surrounding watery solution (no matter whether the ion is a protein or a fatty acid or some complicated lipoid or a complicated carbohydrate or even a crystalloid) can or must give rise to a P.D. which is depressed when a diffusible salt is added to the surrounding watery solution.

The idea that lipoids are the substances responsible for the P.D. of tissues led Beutner to an extensive and most interesting investigation of the P.D. at the boundary of water-immiscible substances and water.<sup>10</sup> He found always a depressing effect of the addition of salt. Beutner

<sup>7</sup> Loeb, J., and Beutner, R., *Biochem. Z.*, 1912, xli, 1.

<sup>8</sup> MacDonald, J. S., *Proc. Roy. Soc.*, 1900, lxxvii, 310.

<sup>9</sup> Loeb, J., and Beutner, R., *Biochem. Z.*, 1913, li, 288; 1914, lix, 195.

<sup>10</sup> Beutner, R., *Die Entstehung elektrischer Ströme in lebenden Geweben*, Stuttgart, 1920.

tried to explain this on the basis of differences in the electrolytic dissociation in the watery and the water-immiscible (oily) phase. Such an explanation cannot be applied to the experiments with protein solutions and yet these latter solutions also show the depressing effect of the addition of salt on the P.D. in a most striking way. In this latter case the depressing effect of the salt on the P.D. is due to the Donnan equilibrium and there is no reason why the theory of membrane equilibria should not apply to the P.D. between oily and watery phases since this theory only demands that one ion of the oily phase should be prevented from migrating into the watery phase. Any lipid ion would fulfill this postulate of the theory. The peculiarities of electrolytic dissociation found by Beutner in non-aqueous solutions must, however, influence the Donnan equilibrium in a secondary way since this equilibrium depends on ionization.

#### SUMMARY AND CONCLUSIONS.

1. When a solution of a salt of gelatin or crystalline egg albumin is separated by a collodion membrane from a watery solution (free from protein) a potential difference is set up across the membrane in which the protein is positively charged in the case of protein-acid salts and in which the protein is negatively charged in the case of metal proteinates. The turning point is the isoelectric point of the protein.

2. Measurements of the pH of the (inside) protein solution and of the outside watery solution show that when equilibrium is established the value pH inside minus pH outside is positive in the case of protein-acid salts and negative in the case of metal proteinates. This is to be expected when the P.D. is caused by the establishment of a Donnan equilibrium, since in that case the pH should be lower outside than inside in the case of a protein-acid salt and should be higher outside than inside in the case of a metal proteinate.

3. At the isoelectric point where the electrical charge is zero the value of pH inside minus pH outside becomes also zero.

4. It is shown that a P. D. is established between suspended particles of powdered gelatin and the surrounding watery solution and that the sign of charge of the particles is positive when they contain gelatin-acid salts, while it is negative when the powdered particles contain metal gelatinate. At the isoelectric point the charge is zero.



5. Measurements of the pH inside the powdered particles and of the pH in the outside watery solution show that when equilibrium is established the value pH inside minus pH outside is positive when the powdered particles contain a gelatin-acid salt, while the value pH inside minus pH outside is negative when the powdered particles contain Na gelatinate. At the isoelectric point the value pH inside minus pH outside is zero.

6. The addition of neutral salts depresses the electrical charge of the powdered particles of protein-acid salts. It is shown that the addition of salts to a suspension of powdered particles of gelatin chloride also diminishes the value of pH inside minus pH outside.

7. The agreement between the values 58 (pH inside minus pH outside) and the P. D. observed by the Compton electrometer is not only qualitative but quantitative. This proves that the difference in the concentration of acid (or alkali, as the case may be) in the two phases is the only cause for the observed P.D.

8. The Donnan theory demands that the P.D. of a gelatin chloride solution should be  $1\frac{1}{2}$  times as great as the P.D. of a gelatin sulfate solution of the same pH and the same concentration (1 per cent) of originally isoelectric gelatin. This is found to be correct and it is also shown that the values of pH inside minus pH outside for the two solutions possess the ratio of 3:2.

9. All these measurements prove that the electrical charges of suspended particles of protein are determined exclusively by the Donnan equilibrium.



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## THE THEORETICAL RESPONSE OF LIVING CELLS TO CONTACT WITH SOLID BODIES.

By WALLACE O. FENN.

(*From the Laboratory of Applied Physiology, Harvard Medical School, Boston.*)

(Received for publication, December 23, 1921.)

Recent discussions of the behavior of living cells in contact with solid bodies have been published by Loeb (1) and Tait (2). Both authors admit that two factors are involved in the reactions of the cells—changes in consistency of the protoplasm and surface tension forces. Tait lays particular stress on the latter, while Loeb believes that changes in consistency are more important. The latter has, perhaps, taken a somewhat safer position in his interpretation of the phenomena described. It would seem, however, that the relative importance of these two factors must vary with the particular type of cell chosen and the conditions of observation. Thus, invertebrate blood cells are more at the mercy of surface tension forces on coming into contact with a solid body than *Amæba* or mammalian leucocytes. It is certainly true, nevertheless, that all blood cells are subjected to the abnormal forces of surface tension when they strike a foreign body. Whether or not these forces are sufficient to determine the behavior of the cell depends upon its fluidity. If it is only to enable us to discuss intelligently the relative importance of consistency changes and surface tension forces as applied to cells, it is necessary to formulate these hypotheses as accurately as possible.

There is, unfortunately, little to be said about such an indefinite factor as changes in the consistency of protoplasm, but surface tension lends itself readily to quantitative treatment. Tait has endeavored to predict the behavior of cells in contact with flat surfaces and small solid bodies from principles of surface tension. His discussion, however, was both incomplete and, in respect to phagocytosis of small bodies, erroneous. It seems important, therefore, to amplify and correct his discussion of the theory.

To begin with a simple case, we may consider the various positions which a perfectly fluid hypothetical cell would assume on a flat surface of glass in terms of the surface tensions between the cell and the plasma, the plasma and the glass, and the glass and the cell. In Fig. 1 let  $a$  represent the cell suspended in plasma,  $P$ , before coming into contact with the glass,  $G$ , and let  $b$  represent the same cell in an equilibrium position with respect to  $G$ . In taking this position or any other position in contact with any solid body,  $G$ , the  $cp$  interface,  $x$ , has been increased (or decreased), and an area,  $s$ , of the  $gp$  inter-

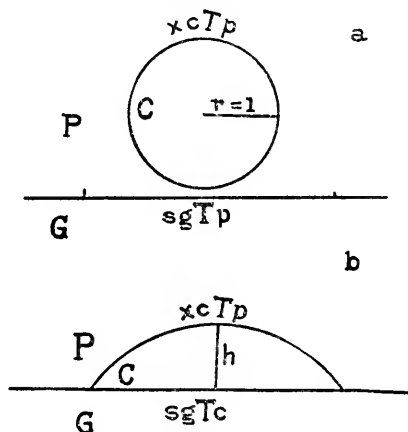


FIG. 1. Diagram of a liquid sphere,  $C$ , representing a cell suspended in plasma  $P$ , before (a) and after (b) coming into contact with a glass surface,  $G$ .  $x$  is the area of the  $cp$  interface;  $cTp$ , the surface tension of the  $cp$  interface;  $s$ , the area of the  $gc$  interface at (b) and the corresponding and equal  $gp$  interface at (a).

face has been exchanged for an equal area of the  $gc$  interface. The surface energy,  $E$ , at  $b$  is expressed by the equation

$$E = xcTp + sgTc - sgTp \quad (1)$$

where  $cTp$ ,  $gTc$ , and  $gTp$  represent the surface tensions of the  $cp$ ,  $gc$ , and  $gp$  interfaces respectively.

The problem is to calculate the height,  $h$ , of the cell above the glass at equilibrium in terms of  $gTc$ ,  $gTp$ , and  $cTp$ , the volume of the cell remaining constant. Now by definition the surface energy,  $E$ , at equilibrium must be at a minimum, and  $\frac{dE}{dh} = 0$ . To find  $\frac{dE}{dh}$  let us



express  $x$  and  $s$  in equation (1) in terms of  $h$  when the radius,  $r$ , of the cell (when spherical as at  $a$ ) is equal to 1. In order to do this it is necessary to neglect the effect of gravity and assume that  $C$  always has the shape of a sphere or spherical segment. This is quite legitimate since the effect of gravity would be practically negligible where the dimensions of  $C$  are those of leucocytes and where the difference in density between  $C$  and  $P$  is small.<sup>1</sup> By familiar formulas we may write

$$x = \pi (a^2 + h^2) \quad (2)$$

$$s = \pi a^2 \quad (3)$$

where  $a$  is the radius of the base of the cell.

The volume of the spherical segment at  $b$  equals the volume of the sphere at  $a$  (Fig. 1) or

$$\frac{1}{6} \pi h (h^2 + 3a^2) = \frac{4}{3} \pi r^3$$

Putting  $r = 1$  and solving

$$a = \sqrt{\frac{8}{3h} - \frac{h^2}{3}} \quad (4)$$

Substituting the value of  $a$  from equation (4) in (2) and (3) and introducing the resulting values of  $x$  and  $s$  in equation (1) we have

$$E = cT\dot{p} \left( \pi h^2 + \frac{8\pi}{3h} - \frac{h^2\pi}{3} \right) + (gTc - gT\dot{p}) \left( \frac{8\pi}{3h} - \frac{\pi h^2}{3} \right) \quad (5)$$

Putting  $n = cT\dot{p}$  and  $m = gTc - gT\dot{p}$ , differentiating with respect to  $h$ , and simplifying, we find

$$\frac{dE}{dh} = \frac{\pi h^3 \left( 2n - \frac{2}{3} (m+n) \right) - \frac{8\pi}{3} (m+n)}{3h^2} \quad (6)$$

At equilibrium  $\frac{dE}{dh} = 0$ . Hence, putting the right hand member of equation (6) equal to 0 and solving for  $h$ , we have

<sup>1</sup> Gravity would merely shift the equilibrium point without altering the principles involved.

$$h = \sqrt[3]{\frac{8(m+n)}{4n-2m}} = \sqrt[3]{\frac{8(gTc - gTp + cTp)}{4cTp - 2gTc + 2gTp}} \quad (7)$$

With this equation, knowing  $m$  and  $n$ , we can calculate  $h$  in terms of the original radius of the cell and from  $h$ , in turn, we can calculate by equation (4) the radius of the base of the cell,  $a$ .

Another formula for the equilibrium is given by Tait from considerations of the contact angle in Fig. 2.

$$gTp = gTc + cTp \cos A \quad (8)$$

$$\begin{aligned} gTp - gTc &= cTp \cos A \\ -m &= n \cos A \end{aligned} \quad (9)$$

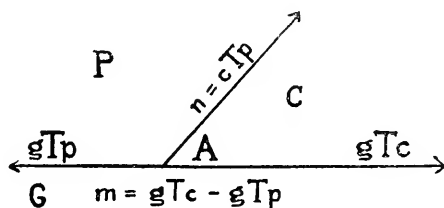


FIG. 2. Diagram of the equilibrium of surface tension forces at the angle of contact,  $A$ , between a hypothetical cell,  $C$ , suspended in plasma,  $P$ , and a glass slide,  $G$ . Arrows indicate direction of pull only.

By simple geometry and trigonometry it may be shown that

$$\cos A = \frac{4 - 2h^3}{4 + h^3} \quad (10)$$

Substituting the value of  $h$  from equation (7) in (10) and reducing, we have  $\cos A = -\frac{m}{n}$ , which is the same as equation (9), and *proves that the same equilibrium results either from considerations of the contact angle or considerations of the surface energy.*

Now Tait's argument is incomplete because in using equation (9) he assumes that  $A = 0$ , *i.e.*, that the cell spreads to infinity, whence  $\cos A = 1$  and  $m + n = 0$  or, in his terminology, a cell will spread to infinity<sup>2</sup> when

<sup>2</sup> The cell is, of course, assumed to be a mathematical sphere without structure. Even a pure liquid could not spread out in a layer less than one molecule thick.

$$gTc - gTp + cTp = \text{or} < 0$$

But equation (7) as well as equation (9) proves that a cell may spread without spreading to infinity; indeed, any position is possible. Thus in equation (7) if  $n = +1$  and  $m = +1$ , then  $h = 2$ , *i.e.*, twice the radius of the cell when spherical, and the cell will not spread at all on  $G$ . If  $n = +1$  and  $m = -1$ , then  $h = 0$  and the cell will spread to infinity. If, however, to take an intermediate case,  $n = +1$  and  $m = 0$ , then  $h = \sqrt[3]{2} = 1.26$ , which means that the cell will take the position of a hemisphere, having the same volume as the original sphere. At this point  $\cos A = -\frac{m}{n} = 0$  and  $A = 90^\circ$ .

When  $m = 0$  there is neither gain nor loss of energy when an area of the interface between  $G$  and the plasma is replaced by an equal area of the interface between  $G$  and the cell. The explanation of the hemisphere as the equilibrium shape under these circumstances (when  $m = 0$ ) is that in this position the surface of the cell exposed to the plasma is at a minimum. This brings out the significant fact that the area of the exposed surface of a liquid sphere of diameter,  $d$ , which is spreading to infinity on a flat surface, first decreases, passes through a minimum when the apparent diameter (diameter of the base) is  $1.26d$ , then increases until, at an apparent diameter of  $1.86d$ , it is again equal to the original surface area, and finally increases to infinity. Exactly comparable changes in the surface area of the cell occur during the ingestion of a small particle, except that the final increase is limited by the size of the particle instead of by infinity. This is clearly the reason why adhesiveness is such a familiar property of blood cells. We have thus been led to a definition of what we mean by adhesiveness or stickiness of cells. *A cell which is stuck to a slide is one that is incompletely spread out by forces of surface tension.* The energy necessary to detach the cell is stored up as surface energy on the newly formed surfaces. If the cell tears, leaving a layer of protoplasm still clinging on the slide, we have an exception in which the energy expended is merely a measure of the cohesion of the protoplasm (surface tension between protoplasm and protoplasm). It is of course possible that the natural rigidity of a cell will prevent it from spreading out on a solid surface so far and, therefore, from

sticking as hard as it otherwise would, but it is always true that the force which holds a cell to a glass slide, indeed which holds any liquid to any solid surface, is the force of surface tension.<sup>3</sup>

In extending this discussion to cover cases where the surface of  $G$  is curved and  $G$  becomes a small sphere, Tait makes a fundamental error. He argues that a particle of  $G$  will be ingested by  $C$  if the surface energy can thereby be decreased. In other words,  $G$  will be ingested if the decrease in energy, due to exchanging  $sgTp$  for  $sgTc$  ( $-sm$ ), more than compensates for the increase in energy due to the enlargement of the  $cp$  interface ( $\Delta xn$ ) after ingestion of  $G$ . Thus a particle will be ingested if  $-sm > \Delta xn$  or if  $sm + \Delta xn < 0$ .

For the comparison with the condition necessary for the spreading of a cell on a flat surface, that  $m + n =$  or  $< 0$ , Tait puts this inequality into the form  $m + \frac{\Delta x}{s}n < 0$  and reasons that since  $\frac{\Delta x}{s}$  is

“as a rule”<sup>4</sup> less than 1,  $m + \frac{\Delta x}{s}n$  will be more likely to be less than 0

than will  $m + n$ . Hence he predicts that if a cell ingests a small particle of  $G$  it will surely spread on a flat surface but that the reverse may or may not be true.

This prediction is erroneous, because, as we shall attempt to show, even though the surface energy may be less at complete ingestion than before ingestion, *it is always at a minimum (still less) at incomplete ingestion*, unless  $m + n = 0$ ; i.e., unless the cell would spread to infinity on a flat surface. This means that no particle of  $G$  can ever be completely ingested by  $C$  unless  $C$  will spread to infinity on  $G$ .

The truth of this statement becomes evident from considerations of the contact angle between  $C$  and  $G$ . As  $G$  becomes more and more nearly ingested the angle of contact approaches 0. It can never reach 0 unless  $-\frac{m}{n} = 1$  for from equation (9)

<sup>3</sup> Some writers (Mathews, A. P., *Physiol. Rev.*, 1921, i, 553) would restrict the term surface tension to the free energy of cohesion on liquid surfaces. It is used here to denote the intensity factor of the free surface energy on either solid or liquid surfaces regardless of the nature of the forces involved.

<sup>4</sup> Actually it is always less than 1. It approaches 1 as a limit as the diameter of the ingested particle approaches infinity.

$$\cos A = -\frac{m}{n} = \cos 0 = 1$$

Since this subject has apparently never been worked out from the point of view of surface energy it seemed desirable to be satisfied that, when the forces of surface tension at the contact angle are in equilibrium, the surface energy is also at a minimum. Unfortunately, we have not been able, even with the expert assistance of a professional mathematician, to express  $x$  and  $s$  of equation (1) in terms of the degree of ingestion of a particle as measured by the length of the line  $y$  in Fig. 3, and thereby to obtain the value of  $\frac{dE}{dy}$  and finally an equation

like (7) for a surface of any degree of curvature. Theoretically this procedure is not impossible, but the necessary equations are too difficult to solve. A test case has been taken, however, where the radius of the particle,  $g$ , is one-quarter of the radius of the cell ( $r = 1$ ) and the values of  $\Delta x$  (the change in surface area of the cell in contact with the plasma), and of  $s$  (the surface area of  $G$  in contact with  $C$ ), have been calculated for the different values of  $y$ , the height of the spherical segment inside the cell (Fig. 3).<sup>5</sup> These values are given in Table I together with the cosine of the corresponding angle of contact. By assigning various values to  $m$  and  $n$  and using the calculated values of  $\Delta x$  and  $s$  in the equation<sup>6</sup>  $sm + \Delta xn = E$ , curves can be plotted showing how the surface energy,  $E$ , varies as  $y$  increases to 0.5 or twice the radius of the particle,  $G$ ; *i.e.*, as ingestion approaches completion. These curves, for different values of  $m$  when  $n = 1$ , are shown in Fig. 4. The values of  $m$  used were calculated from the equation for the contact angle equilibrium,  $m = -n \cos A$  where  $A$  is the angle of contact corresponding to the chosen value of  $y$  and  $n$

<sup>5</sup> The complication arising from the increase in the radius of the cell to  $r + \Delta r$ , as the particle is more and more completely ingested is responsible for the difficulty of the calculation. In order to evaluate  $\Delta x$  and  $s$  it is necessary to solve for  $z$  (Fig. 3) in an equation involving  $z^3$  and  $z^2$  by trial and error. This must be done to four significant figures in order to calculate  $\Delta x$  and  $s$  accurately enough for the purpose.

<sup>6</sup> This equation is the same as (1), replacing  $gTc - gTp$  by  $m$  and  $cTp$  by  $n$  as in equation (6) and omitting the constant  $4\pi r^2 n = 12.566n$  ( $x - \Delta x$ ) $n$  representing the surface energy of the cell when spherical.

= 1. On each curve the point corresponding to the value of  $\cos A$  (Table I) used in calculating  $m$  for that curve is marked by a circle.<sup>7</sup> The fact that the surface energy is always at a minimum at the marked point proves that, when the forces of surface tension are in equilibrium at the contact angle, the surface energy of the whole system is also at a minimum. That part of each curve beyond the minimum is dotted to indicate that it is imaginary, because the cell would continue to ingest a particle only as long as the surface energy was decreasing.

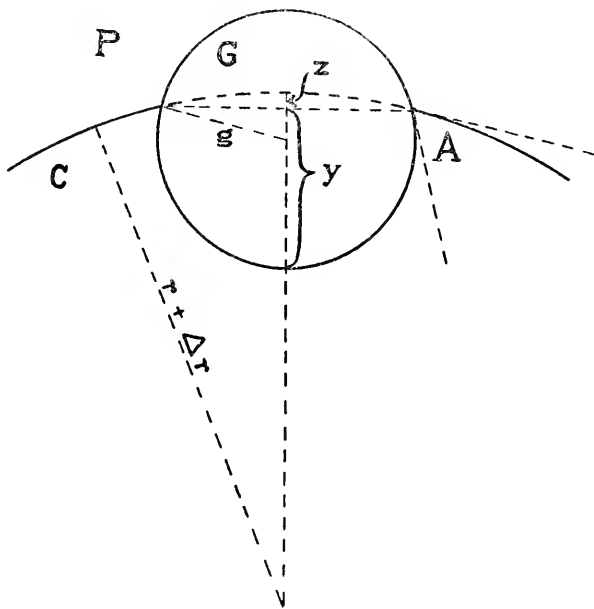


FIG. 3. Diagram showing geometrical construction used in calculating surface areas involved, as a solid spherical particle of radius  $g$  is ingested by a liquid sphere (shown in part) of radius  $4g$  or  $r$ .  $A$  is angle of contact.

The error of Tait's statement and of the predictions based upon it is evident from the figure. The minimum surface energy is not

<sup>7</sup> By simple geometrical and trigonometrical consideration of Fig. 3 it is found that

$$\cos A = \frac{(r + \Delta r)^2 + g^2 - (r + \Delta r + g - z - y)^2}{2g(r + \Delta r)}$$

This equation was used in calculating  $\cos A$  as given in Table I.

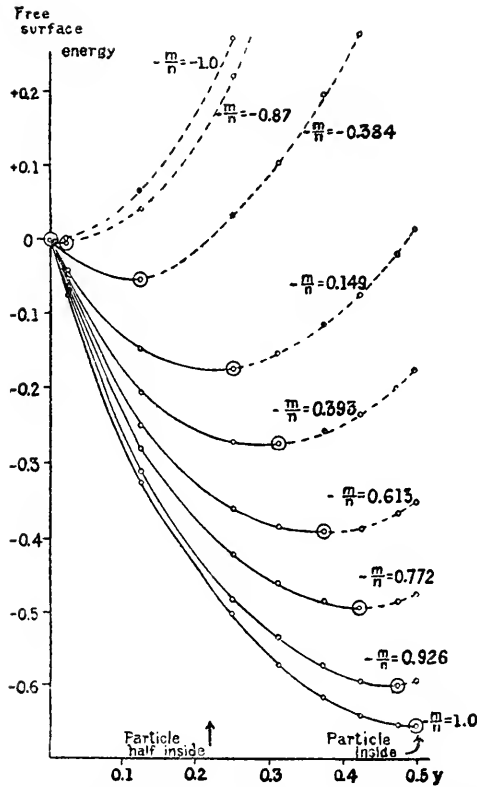


FIG. 4. Graphs showing how free surface energy (expressed in relative not absolute units) varies during the passage of a solid particle of radius 0.25, inside a hypothetical cell of radius 1.0, for various values of  $\frac{m}{n}$  where  $n$  and  $m$  represent the gain in free surface energy caused by a given increase in the area of the cell plasma and cell glass interfaces respectively. Abscissæ,  $y$ , vary with the position of the particle with respect to the cell, half inside at 0.22 $y$  and completely inside at 0.5 $y$ . Points marked by large circles are points of equilibrium where surface energy is at a minimum, and represent also positions of cell and particle where the values of  $m$  and  $n$  used in calculating that curve would be in equilibrium at the angle of contact. The curves are dotted beyond these points to indicate that work must be done upon the particle to complete its ingestion since the surface energy is increasing. Values of  $-\frac{m}{n} = \cos A$  are given in Table I. See text for further discussion.

reached at complete ingestion until the surface tension is such that the cell will spread to infinity. In the lower five curves in Fig. 4 the surface energy is less at complete ingestion than before ingestion; *i.e.*, Tait's condition is fulfilled, but the particle is nevertheless only partially ingested except in the lowest curve.

From this point of view the surface of every particle, however small, must be regarded as an infinite surface, just as a flat surface may be regarded as a curved surface with a radius infinitely large. The only predictions which we can make from principles of surface tension are:

1. If a cell spreads to infinity on a flat surface of  $G$ , it will completely ingest a particle of  $G$ .
2. If a cell does not stick to a flat surface of  $G$ , a small particle of  $G$  will not stick to the cell.
3. If a cell is partially spread out on a flat surface of  $G$ , a particle of  $G$  will be partially ingested.

Tait has predicted from his discussion that: "If a substance,  $C$ , is unstable on a slab of  $G$ , then  $C$  will ingest  $G$ ." This is true if "unstable" means spreading to infinity; but when he tries to prove this prediction experimentally by observing that hyaline thigmocytes are unstable on glass and ingest small particles of glass, it may fairly be objected that he could not have observed the cells spreading to infinity and that the prediction consequently is not fulfilled by the facts. The explanation of the experiment is either that the cell was too rigid to spread to infinity on a flat surface though it could do so readily on the curved surface of a small particle, or that the equilibrium observed on a flat surface was a true surface tension equilibrium and that the small force of surface tension which prevented the small particle from being completely ingested was easily overcome by the ameboid movement (changes in consistency) of the cell.

A similar explanation may be given when Tait predicts that: "If  $C$  ingests a particle of  $G$ , then  $C$  (may or) may not be unstable on a slab of  $G$ ," and adds in consequence that "the fact that stable cells such as mammalian polymorphonuclears do not spread extensively on glass is no evidence that they ingest by other than physical means." The true prediction from principles of surface tension is that if  $C$  ingests  $G$ , it will spread to infinity on a slab of  $G$ , and the ob-



served behavior of leucocytes means either that the rigidity of the cell prevents its reaching a true equilibrium on a flat surface, or that it ingests, by its ameboid movement, a small particle of  $G$  which otherwise would be merely stuck on the outside. Both alternatives seem equally probable. Obviously such tests of the surface tension hypothesis are worthless.

TABLE I.

*Areas of Contact Surfaces during Ingestion of a Particle.*

$y$	$s$	$\Delta x$	$\cos A$
0.5	0.785	+0.131	1.00
0.475	0.746	+0.092	0.926
0.425	0.667	+0.024	0.772
0.375	0.588	-0.028	0.613
0.312	0.490	-0.082	0.393
0.250	0.393	-0.118	0.149
0.125	0.196	-0.130	-0.384
0.025	0.039	-0.037	-0.870
0	0	0	-1.0

$y$  expresses the degree of ingestion as shown in Fig. 3. Ingestion is complete at  $0.5y$ , one-half complete at  $0.22y$ .  $s$  is the area of the cell in contact with the particle;  $\Delta x$  the change in the area of the cell plasma interface;  $A$  is the angle of contact.

Is it easier to ingest a small particle than a large particle, and if so, why? Tait says: "If in the case of a given phagocytic cell we have a certain volume of material which can just be ingested as a spherical piece, that cell will ingest the same volume more readily when the material is either sub-divided or distorted from the spherical form for by this means  $s$  is increased without change in  $x$  [ $\Delta x$  in our terminology]." It is true from principles of surface tension that a large particle will be less completely ingested than a small one for the same values of  $m$  and  $n$ , *i.e.*, for the same contact angle, but the same limiting values of  $m$  and  $n$  (*i.e.*,  $-\frac{m}{n} = 1$ ) are necessary for complete ingestion of any particle of any shape or size. It is true, however, that a small particle could be more easily pulled inside against the force of surface tension from some equilibrium position in the surface than

a large particle which made the same angle of contact with the cell. Also, it is easier for a cell to spread around a small particle than around a large one because the necessary mechanical deformation is less.

Phagocytosis has been described repeatedly as taking place in two stages (Kite and Wherry (3)), the actual ingestion being preceded by a phase in which the object is merely stuck on the outside. The preliminary stage is clearly a surface tension phenomenon. The frequency of its occurrence is due to the fact that the surface of the cell exposed to the plasma is thereby decreased.<sup>8</sup> When objects seem to be permanently stuck on the outside of a cell this may be a true surface tension equilibrium or, more likely, it may be that the rigidity of the structure of the interior of the cell prevents the further deformation necessary to reach a true surface tension equilibrium with the object completely inside.

In conclusion emphasis may be laid upon the significance, from the point of view of surface tension of Tait's general proposition which states that only unstable cells tend to be phagocytic.

#### SUMMARY.

The theoretical behavior of a hypothetical fluid cell in contact with flat and curved solid surfaces is discussed from the point of view of surface tension.

An equation is derived for calculating the equilibrium position of the cell on a flat surface in terms of the surface tensions between the cell and the plasma, the plasma and the solid surface, and the solid surface and the cell. It is shown that the same equilibrium is predicted from consideration of the contact angle between the cell and the solid body.

The relative surface energy has been calculated at various stages in the ingestion of a solid particle by a fluid cell four times as large in diameter, and it is thus shown that no particle will be ingested until the surface tensions are such that the cell would spread to infinity on a flat surface of the same substance. Here again the same equilibrium is predicted from considerations of the contact angle.

<sup>8</sup> This decrease is evidenced in Table 1 by the negative values of  $\Delta x$  when  $y$  is 0.375 or less.

The adhesiveness of blood cells to solid substances is shown to be a pure surface tension phenomenon, but in most reactions between living cells and solid bodies the fluidity of the protoplasm is also a factor of prime importance.

The frequent occurrence of adhesiveness as a property of cells in contact with solid bodies is due in part to the fact that, by so adhering, the surface area of the cell not touching the solid is decreased.

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# CHANGE OF ACID AGGLUTINATION OPTIMUM AS INDEX OF BACTERIAL MUTATION.

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## INTRODUCTION.

Two distinct varieties of microbe have been shown to exist in cultures of the bacillus of rabbit septicemia (1). These have been designated as Microbes D and G. Microbe D is the variety isolated from rabbits dead of spontaneous infection with the rabbit septicemia bacillus. It is characterized by diffuse growth in serum and plain broth, forms opaque fluorescing colonies on serum agar, and is highly virulent for rabbits. Microbe G, first discovered accidentally in Microbe D cultures, has been proved to be a true mutant of the parent D form (2). The mutant Microbe G grows in granular fashion in liquid media, forms translucent bluish colonies with no fluorescence, and exhibits extremely low virulence for rabbits. The mutation experiments demonstrating that Microbe D, under controllable conditions, changes into Type G were performed with D strains arising from single individuals isolated by Barber's pipette.

The granular growth of Microbe G in fluid medium is one of its most striking differential characters, and has persisted throughout transplants for more than 1 year. This sedimenting growth of Type G in broth, compared to the evenly suspended, uniformly turbid appearance of broth cultures of Type D led to an examination of the acid agglutination optima of the two types.

## *Methods.*

The method for the determination of the acid agglutination optimum was that of Michaelis (3), later described in full by Beniasch (4). It consisted in mixing carefully prepared suspensions of the organism

to be tested with equal volumes of buffer mixtures of varying  $\text{CH}^+$ . Two buffer series were employed, Na lactate-lactic acid and Na acetate-acetic acid. The mixtures were made according to Tables I and II.

TABLE I.  
*Na Lactate-Lactic Acid Series.*

pH	4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Na Lactate N/10.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lactic acid N/10.....	0.06	0.12	0.25	0.5	1.0	—	—	—	—
Lactic acid N.....	—	—	—	—	—	0.2	0.4	0.8	1.6
Distilled water.....	1.54	1.48	1.35	1.1	0.6	1.4	1.2	0.8	—

TABLE II.  
*Na Acetate-Acetic Acid Series.*

pH	5.6	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	Final vol- ume, 2.1 cc.
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	
Na Acetate N/10.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Acetic acid N/10.....	0.06	0.12	0.25	0.5	1.0	—	—	—	—	
Acetic acid N.....	—	—	—	—	—	0.2	0.4	0.8	1.6	
Distilled water.....	1.54	1.48	1.35	1.1	0.6	1.4	1.2	0.8	—	

### *Preparation of Suspensions.*

Microbe G sediments rapidly in fluid media. It fails to remain in even suspension when the sediment from a centrifuged culture is taken up in 0.85 per cent NaCl. If, however, such sediments are repeatedly washed in large volumes of distilled water, very stable suspensions can be obtained. In order to secure perfect comparability, Microbe D was treated in a similar manner, although in this case washing with distilled water is unnecessary and it yields stable suspensions in 0.85 per cent NaCl. The technique of preparation of suspensions was as follows.

5 per cent rabbit serum broth cultures, 24 hours incubation, were centrifuged, the supernatant fluids discarded, and the sediments thoroughly shaken in a volume of distilled water equal to that of the

original culture. The centrifugation and resuspension in distilled water were repeated four times in all. The final suspensions were carefully brought to a uniform turbidity.

## EXPERIMENTAL.

All experiments were carried out by adding 1 cc. of distilled water suspension of the microbe in question to an equal volume of each of the buffer mixtures just described. The tubes were carefully shaken, placed in the water bath at 43°C. and readings taken at 1, 2, and 16 hours.

TABLE III.

*Acid Agglutination Optimum of Microbes D and G, Strain R 15.  
Na Lactate-Lactic Acid Buffer Series.*

Organism.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH. Final reading.
	pH	4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4	
Microbe D.	1 hour.	0	0	0	0	+	+	0	0	0	3.5-3.3
	2 hours.	0	0	0	0	++	++	+	+	Tr.	
	16 "	0	0	0	0	C	C	+	Tr.	Tr.	
Microbe G.	1 hour.	Tr. +	+	+	++	+	0	0	0	0	4.7-3.8
	2 hours.	Tr. +	++	++	C	++	0	0	0	0	
	16 "	C	C	C	C	+	Tr. +	0	0	0	

In this and the following tables lesser degrees of flocculation are recorded as ++, +, and Tr. (trace). C indicates complete flocculation.

The agglutination optimum was considered to be that zone of  $C_{H^+}$  where complete flocculation occurred; that is, where the microbes sedimented so perfectly as to leave a water-clear supernatant fluid. Lesser degrees of agglutination are recorded as ++, +, and Tr. (trace). The readings are greatly facilitated by holding the tubes before a powerful beam of light, projected downward in front of a dark background.

*Experiment 1. Acid Agglutination Optimum, Microbes D and G, Strain R 15, in Na Lactate-Lactic Acid Buffer Series.*—Suspensions of Microbes D and G, isolated from Strain R 15, bacillus of rabbit septicemia, were tested against the Na lactate-lactic acid buffer series. The experiments were carried out as described above. The results are recorded in Table III.

The results recorded in Table III show a definite difference in acid agglutination optimum of the two varieties. The readings, taken at 1, 2, and 16 hours, indicate that the reaction does not take place with the speed of that of immune agglutination of the majority of bacteria. It is necessary to allow ample time to elapse before taking the final readings. 16 hours have been found to be sufficient, no material change in readings being noted after this time.

The same suspensions were tested against the Na acetate-acetic acid buffer series, with a similar result recorded in Table IV. Temperature, as before, was 43°C. in the water bath.

TABLE IV.  
*Acid Agglutination Optimum of Microbes D and G.  
Na Acetate-Acetic Acid Series.*

Organism.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH.
	pH	5.5	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	
Microbe D.	1 hour.	0	0	0	0	0	0	0	+	+	3.5-3.2
	2 hours.	0	0	0	0	0	0	0	C	C	
	16 "	0	0	0	0	0	0	0	C	C	
Microbe G.	1 hour.	0	0	0	+	++	++	+	0	0	4.75-3.8
	2 hours.	0	0	0	++	C	C	++	0	0	
	16 "	Tr. +	Tr.	++	C	C	C	C	+	Tr.	

The results in the case of the Na acetate-acetic acid series correspond to those of the Na lactate-lactic acid mixtures. The final readings are identical, the only difference lying in a slightly more rapid flocculation in the acetate series.

*Variability of Optimum of Microbe G as Compared to that of Microbe D.*

A number of strains were now collected, the D and G types isolated and subjected to test with the Na acetate-acetic acid series. The result (Table V) confirms the findings of the previous experiments. Final reading was made after 16 hours with incubation at 43°C. The results are presented in Table V.



It will be noted that the acid agglutination optimum for Microbe D is the same in case of all three of the strains tested. On the other hand, the optimum for Microbe G varies to a considerable extent. This variation is never so great as to prejudice its value as a criterion of differentiation from the parent D form. In all cases complete flocculation of Type G occurs at a distinctly lower  $C_H^+$  than that of Type D. The difference between the two types in regard to the smallest amount of hydrogen ion in which complete flocculation takes place is never less than 0.6 pH. In short, the organism in the process of mutation gains in sensitivity to flocculation in the presence of H ions.

TABLE V.

*Acid Agglutination Optima of D and their Mutant G Forms.  
Na Acetate-Acetic Acid Series.*

Strain.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH.
	pH	5.6	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	
R 15	D-S 49	0	0	0	0	0	0	0	C	C	3.5 -3.2
	G-S 52	Tr.	Tr.	++	C	C	C	C	+	Tr.	4.75-3.8
	G-S 28*	Tr.	Tr.	C	C	C	C	C	++	++	5.05-3.8
R 11	D-S 43	0	0	0	0	0	0	0	C	C	3.5 -3.2
	G-S 42	0	0	+	+	+	C	C	C	+	4.1 -3.5
R 22	D-S 31	0	0	0	0	0	0	Tr.	C	C	3.5 -3.2
	G-S 32	0	Tr.	+	+	C	C	C	++	Tr.	4.4 -3.8

\* G-S 28, a mutant from the same parent D strain as G-S 52.

Sobernheim and Seligmann (5) found a strain of *Bacillus enteritidis* to separate into two races. Beniasch (4) tested the acid agglutination point of this organism and found it to have altered its acid agglutination optimum when tested on two different occasions, a year having elapsed between the two tests. In this work apparently no attempt was made to establish the occurrence of a mutation, or to separate the two varieties.

*Variations in the Agglutination Optimum of Type G.*

Table V indicates that the agglutination optima of various strains of Microbe G are not as strictly uniform as those of the parent D type. One of the causes of this variation is passage of the microbe through the animal body. An example of this variation was observed during an attempt to cause reversion of Microbe G to the parent D form.

The Type G strain in question was characteristically of very low virulence. 1.0 cc. of a serum broth culture, injected intrapleurally, was required to produce fatal infection of a 600 gm. rabbit. The organism recovered at necropsy of this animal was cultured and injected into a second animal, and so on. At the third animal passage

TABLE VI.

*Effect of Animal Passage on Acid Agglutinability of Type G.*

	pH	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2
Microbe R 15 G	Before passage.	Tr.	Tr.	++	C	C	C	C	+	Tr.
	After three animal passages.	C	C	C	C	C	C	C	C	C

the virulence had greatly increased,  $10^{-4}$  cc. of a serum broth culture being fatal. But the organism, far from returning to the uniformly turbid growth character of the Type D form, became more intensely granular in its growth. This characteristic was so marked that difficulty was experienced in preparing the washed suspensions for acid agglutination test.

The acid agglutination reaction of Type G strain after animal passage was compared with the same strain which had been transplanted in parallel in serum broth. The Na acetate-acetic acid buffer series was used. The culture was incubated at 43°C. for 16 hours (Table VI).

It will be seen from Table VI, first, that much less hydrogen ion is required to produce complete flocculation, and second that the optimum is very greatly broadened. It has been widened from

pH 4.7 to 3.8 before animal passage, to pH 5.6 to 3.2 after passage through three rabbits.

Up to the present, the change in acid agglutination optimum that occurs during mutation has been accompanied invariably by a great loss in virulence. For example, all Type D strains tested have been fatal to rabbits in doses of  $10^{-5}$  to  $10^{-7}$  of a serum broth culture. The Type G forms arising from such strains are seldom fatal in 0.5 cc. of undiluted culture. Frequently rabbits are able to resist 1.0 cc.

The experiment just described indicates that the decrease in stability to acid does not necessarily go hand in hand with loss of virulence, and certainly bears no causal relationship to such loss. For, while the stability to the hydrogen ion had greatly *decreased* during animal passage, the virulence had *increased* from 0.5 cc. to  $10^{-4}$  cc.

#### SUMMARY AND CONCLUSIONS.

A distinct difference in acid agglutination optimum for Type D (bacillus of rabbit septicemia) and its mutant form, Type G, has been observed. The optimum for Type D lies between pH 3.5 and pH 3.0. This changes during mutation, the resulting Type G mutants having in general an optimum lying between pH 4.7 and pH 3.8.

The constancy of the optimum for Type D is very strict, while that for Type G is slightly less so. The variation is never so great as to cause an overlapping of optima and consequent failure of differentiation.

These acid agglutination optima are in the nature of physical constants for the two types and would imply a fundamental difference in the chemical constitution of the organisms.

Animal passage, far from causing a reversion of the mutant Type G to the primordial Type D form, brings about a still greater instability in the presence of H ions.

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# THE MECHANISM OF GRANULAR GROWTH OF RABBIT SEPTICEMIA BACILLUS TYPE G.

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## INTRODUCTION.

It has been stated by Beniasch (1) that the acid flocculation optimum of bacteria is referable only to the  $C_H^+$  and is not influenced by the unionized acid or the anion of the acid. This concept may hold for buffer mixtures made up of certain acids and their sodium salts. But certain buffer mixtures exist which are composed of an acid plus some totally different substance, bearing no close chemical relationship to the acid. Examples of such buffer mixtures are glycoll-HCl and glycoll-Na acetate- $NaH_2PO_4$ -HCl. These have been found by the writer (2) to be very useful in acid agglutination studies. It is important to learn whether such buffer substances exert an effect on the acid agglutination point of bacteria, which might differ from the values found for the Michaelis (3) series.

## EXPERIMENTAL.

The Na lactate-lactic acid buffer series of Michaelis (3) covers a range from pH 4.7 to 2.4. It was desired to test the behavior of distilled water suspensions of Microbe G and D in higher  $C_H^+$ . Since this range was not covered by the Na lactate-lactic acid series, recourse was had to the glycoll-HCl series of Sørensen (4), which covers a range from pH 3.0 to pH 1.2. This buffer series was prepared from the Sørensen chart.

The pH of these mixtures was tested colorimetrically and checked by the potentiometer.

The flocculating activity of this buffer series was then compared to that of the Na lactate-lactic acid series. The technique of the

experiments was identical with that described in the preceding paper (5). 1 cc. of the buffer mixtures was added to equal volumes of four times washed distilled water suspensions of Microbes D and G, bacillus of rabbit septicemia. The mixtures were placed in the water bath at 43°C. for 16 hours, and readings taken. The results of this experiment are recorded in Table I.

TABLE I.

*Agglutination of Microbes D and G in Na Lactate-Lactic Acid and Glycocoll-HCl Buffer Series.*

	pH	4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4		Complete agglutination.
Na lactate- lactic acid.	Microbe D.	0	0	0	++	C	C	+	+	0		<i>pH</i> 3.5-3.3 None at 2.7- 2.4. 4.7-4.1 None at 3.3- 2.4.
	Microbe G.	C	C	C	++	+	0	0	0	0		
Glycocoll- HCl.	pH	3.0	2.8	2.6	2.4	2.2	2.0	1.8	1.6	1.4	1.2	
	Microbe D. Microbe G.	C ++	C ++	C +	C +	++ +	++ 0	++ 0	++ 0	++ 0	0 0	3.0-2.8 ++ 3.0-2.8

In this and the following tables the mixtures were kept in the water bath for 16 hours at 43°C. C indicates complete flocculation; Tr., trace.

The results given in Table I indicate that other factors besides the  $C_{H+}$  are important in the interpretation of the acid agglutination point of the organism in question. In the case of Microbe D, Na lactate-lactic acid series, for example, complete flocculation occurs at pH 3.5 to 3.3, slight at pH 3.0, a trace at pH 2.7, and none at all at pH 2.4.

On the other hand, Microbe D in the glycocoll-HCl series, flocculates completely at pH 3.0, 2.8, 2.6, and 2.4. This difference of effect in the two buffer series is illustrated graphically in Fig. 1.

A similar result occurs in the case of Microbe G which flocculates completely at pH 4.7, 4.5, and 4.1, and not at all at pH 3.0 to 2.4, in the Na lactate-lactic acid series. Yet ++ agglutination occurs at

pH 3.0 and 2.8 and + at pH 2.6 to 2.2 of the glycoll-HCl series. It would appear that the glycoll or the anion of the acid has the property of broadening the acid agglutination optima of Microbes D and G, or of shifting the optima toward a zone of higher  $C_{H^+}$ .

These results led to experiments which suggest an explanation for the granular growth of Microbe G in plain broth. Washed suspensions of this organism in distilled water, pH 6.0, do not sediment. On the other hand, rapid sedimentation occurs in plain broth at pH 7.4 to 7.0. This leads to the conclusion that broth contains a constituent which, *per se*, agglutinates the Type G organisms, or which has the property, like glycoll, of shifting their acid agglutination optimum.

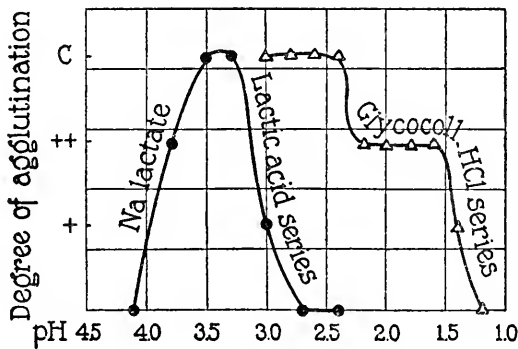


FIG. 1.

Preliminary experiments were made in which the flocculating effect of the various components of plain broth were tested against washed distilled water suspensions of Microbe G. The constituents tested were beef infusion, peptone, and  $Na_2HPO_4$ .

*Beef Infusion.*—500 gm. of chopped beef were extracted in 1,000 cc. of tap water, in the ice box for 16 hours. The mixture was then boiled for 30 minutes, filtered, titrated to pH 7.4, refiltered, and sterilized in the autoclave.

*Peptone Solution.*—10 gm. of Fairchild's peptone were dissolved in 1,000 cc. of distilled water, boiled for 30 minutes, filtered, adjusted to pH 7.4, and sterilized in the autoclave.

*$Na_2HPO_4$ .*—10 gm. of this salt were dissolved in one liter of distilled water adjusted to pH 7.4, and sterilized in the autoclave.

Of these three solutions, at pH 7.4, beef infusion alone showed marked flocculating activity against Microbe G.

The flocculating effect of beef infusion at varying acidities was now tested. The beef infusion solution just described was titrated with N/10 HCl to increasing degrees of acidity, from pH 7.5 to pH 2.0. The beef infusion at all of these  $C_H^+$  was now diluted with distilled  $H_2O$ , the dilutions increasing from 1:2 to 1:40. Each of these dilutions at each pH, was now added in equal volume to 1 cc. of distilled water suspensions of four times washed Microbes D and G. The mixtures were kept in the water bath for 16 hours at 43°C., and readings taken. The results are recorded in Tables II and III.

TABLE II.

*Beef Infusion Agglutination of Microbe G at Varying  $C_H^+$ .*

pH	Amount of beef infusion.							Result.
	1.0 per cent	0.75 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0.075 per cent	
7.5	++	++	++	+	+	0	0	Final volume of beef infusion dilution = 1.0 cc. + distilled $H_2O$ suspension G 1.0 cc.
7.0	C	++	++	++	+	+	0	
6.5	—	C	C	++	++	+	+	
6.0	—	C	C	++	++	++	+	
5.0	—	C	C	C	C	++	+	
4.5	—	C	C	C	C	C	C	
4.0	—	C	C	C	C	C	C	
3.5	—	C	C	C	++	++	+	
3.0	—	C	C	C	++	++	+	
2.5	—	C	C	++	++	+	+	
2.0	—	C	C	++	+	+	+	

Table II shows for Type G, that, as the acidity increases down to to pH 4.5, the amount of beef infusion necessary to cause complete agglutination becomes less and less. At pH 4.5 to pH 4.0 traces of beef infusion cause complete agglutination. This point corresponds to the acid agglutination optimum of Microbe G in various buffer series. Beyond this point, that is, at pH < 4.0, increasing amounts of beef infusion are again necessary to produce complete flocculation.

Table III shows the same effect in the case of Microbe D, the only difference being that complete flocculation of the D type by a given



concentration of beef infusion demands a higher  $C_H^+$  than in the case of Type G. For each organism the range of  $C_H^+$  at which the smallest amount of beef infusion is required for complete sedimentation is precisely the optimum zone of acid agglutination described in the preceding paper.

The results of the experiments recorded in Tables II and III are represented graphically in Fig. 2, in which the pH of the various dilutions of beef infusion are plotted on the abscissæ against the *recip-*

TABLE III.

*Beef Infusion Agglutination of Microbe D at Varying  $C_H^+$ .*

pH	Amount of beef infusion.							Result.
	1.0 per cent	0.75 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0.075 per cent	
7.5	Tr.	0	0	0	0	0	0	Final volume of beef infusion dilution = 1.0 cc. + distilled H <sub>2</sub> O suspension D 1.0 cc.
7.0	+	+	+	Tr.	0	0	0	
6.5	—	C	C	++	+	0	0	
6.0	—	C	C	++	+	Tr.	Tr.	
5.0	—	C	C	C	++	+	+	
4.5	—	C	C	C	C	++	++	
4.0	—	C	C	C	C	C	C	
3.5	—	C	C	C	C	C	C	
3.0	—	C	C	C	++	++	++	
2.5	—	C	C	C	++	++	++	
2.0	—	C	C	++	++	+	+	

*rocals* of the amount of beef infusion on the ordinates. That is, 1.0 cc. of beef infusion is represented by 1, 0.5 cc. by 2, 0.5 cc. by 4, and so on. The points recorded on the graphs are in all cases the amounts of beef infusion which cause *complete flocculation*, represented by C, Tables II and III.

The graphs of Fig. 2 would seem to afford an explanation for the granular growth of Microbe G and the diffuse growth of Microbe D in broth, since at pH 7.0, the  $C_H^+$  at which these organisms are grown, large amounts of beef infusion cause complete sedimentation of Microbe G and little or no agglutination of Microbe D.

What is more, the graphs would appear to indicate that beef infusion, *per se*, does not cause the agglutination. It merely widens the

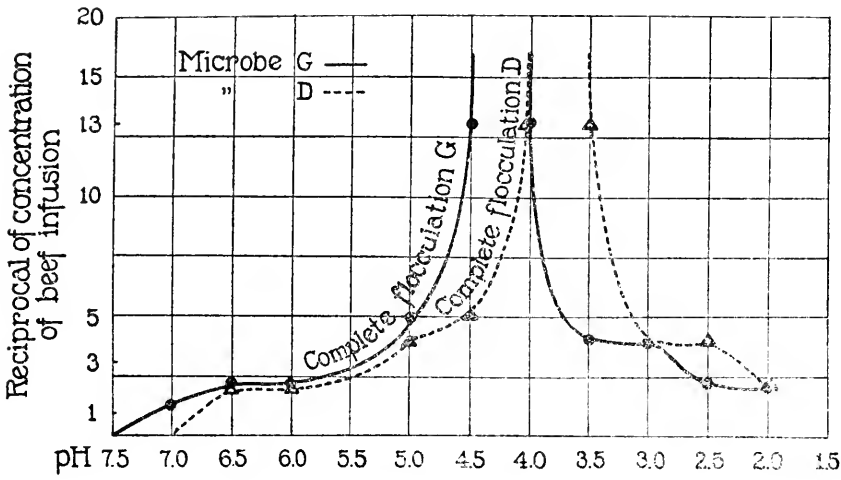


FIG. 2.

acid agglutination optimum zone. This is clear from the fact that small amounts of beef infusion do not cause a flocculation independent of the  $C_{H^+}$ .

Suspensions of Types D and G were similarly tested against decreasing concentrations of peptone (Fairchild) at various  $C_{H^+}$ . The technique of the experiments was identical with that for beef infusion. The peptone solution from which the various dilutions were made

TABLE IV.  
*Peptone Agglutination of Microbe G at Varying  $C_{H^+}$ .*

pH	Amount of peptone.							Result.
	1.0 percent	0.5 percent	0.25 percent	0.2 percent	0.1 percent	0.075 percent	0.05 percent	
7.5-6.0	0	0	0	0	0	0	0	Final volume of peptone dilutions 1.0 cc. + distilled $H_2O$ suspension G 1.0 cc.
5.0	C	++	Tr.	0	0	0	0	
4.5	C	C	Tr.	0	0	0	0	
4.0	C	C	C	C	++	Tr.	0	
3.5	C	C	C	C	C	C	+	
3.0	C	C	C	C	C	C	C	
2.5	C	C	C	C	C	C	C	
2.0	C	+	+	Tr.	0	0	0	

was of 1 per cent concentration in distilled water. The results are recorded in Tables IV and V.

TABLE V.  
*Peptone Agglutination of Microbe D at Varying  $C_{H^+}$ .*

pH	Amount of peptone.							Result.
	1.0 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0.075 per cent	0.05 per cent	
7.5-3.5	0	0	0	0	0	0	0	Final volume of peptone dilutions 1.0 cc. + dis- tilled $H_2O$ suspension D 1.0 cc.
3.0	0	0	C	C	C	C	Tr.	
2.5	0	C	C	C	C	C	C	
2.0	0	0	C	C	++	++	++	

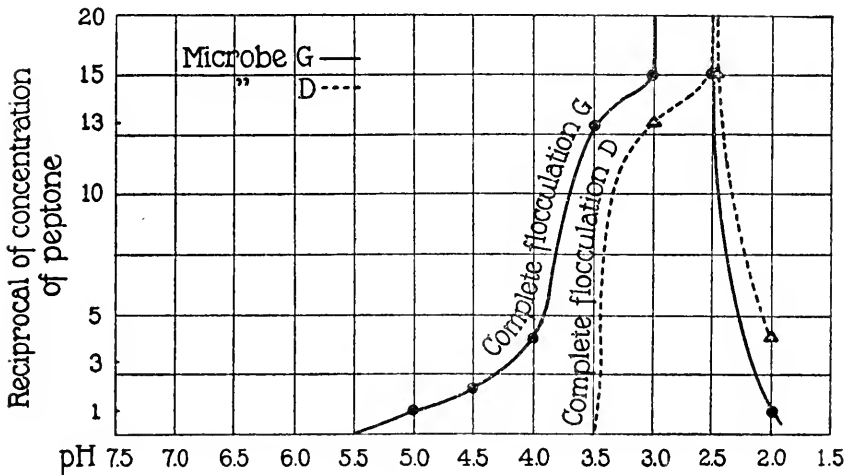


FIG. 3.

It will be observed from Tables IV and V that the results are strikingly different from those obtained with beef infusion. Peptone, contrary to beef infusion, appears to shift the optimum zone of agglutination in the direction of a higher  $C_{H^+}$ , and effect analogous to that observed in the glycocoll-HCl buffer mixtures (Table I).

The optimum for Type G in the lactate and acetate buffer mixtures lies between pH 5.0 and pH 3.8. Peptone changes the optimum to pH 3.0 to 2.5. For Type D, an analogous effect is observed, the

optimum shifting from pH 3.5 to 2.5. The results recorded in Tables IV and V are graphically represented in Fig. 3.

It is interesting to observe that for Microbe D strong concentrations of peptone (0.5 and 0.25 cc. of 1 per cent solution) actually suppress flocculation completely at pH 3.0. The effect of peptone, contrary to that of beef infusion, would appear to be a stabilizing one.

#### SUMMARY.

The acid agglutination optimum of Microbes D and G is not independent of the nature of the buffer mixture. Glycocoll-HCl buffer mixtures cause complete flocculation at high  $C_{H^+}$  (2.7 to 2.4), at which points little or no flocculation occurs with the Na lactate-lactic acid buffer series.

Beef infusion has the property of broadening the acid agglutination optimum of both Microbes D and G, bacilli of rabbit septicemia. This extension is in the direction of a lower  $C_{H^+}$ .

There is no evidence that the beef infusion has the power, *per se*, of agglutinating these organisms. It would seem merely to increase their sensitiveness to sedimentation in the presence of H ions.

The data presented explain the mechanism of the granular growth character of Microbe G in liquid media as compared to the diffuse growth of Microbe D.

Peptone (Fairchild), contrary to beef infusion, shifts the acid agglutination optimum of Microbes D and G in the direction of a higher  $C_{H^+}$ . Strong concentrations of peptone exhibit an inhibitory effect on the agglutination of Microbe D in the optimum zone.

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## THE AGGLUTINATION OF RED BLOOD CELLS IN THE PRESENCE OF BLOOD SERA.

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The optimum for the agglutination of normal sheep cells in isotonic saccharose solution has been given as pH 4.75.<sup>1</sup> To correct a possible error in the colorimetric measurements originally employed electrometric determinations have been made in a similar series of experiments in which graduated amounts of N/10 to N/40 HCl have been added to suspensions of red blood cells in saccharose solution and measurements made of the reaction of the supernatant fluid from which the cells have been removed 15 to 30 minutes after the addition of acid. The average pH 4.76 of the following values thus found corresponds closely with the result of the colorimetric method: pH 4.55, 4.57, 4.79, 4.90, and 5.03.

Cells sensitized with approximately 10 units of immune rabbit serum at pH 5.3, the optimum for combination of the cells with the immune sensitizer<sup>2</sup> and washed with pure saccharose solution at the same reaction agglutinate most promptly at the following reactions in a series of experiments: pH 5.22 to 5.45, 5.26, and 5.06 to 5.30. The average is pH 5.26. The colorimetric method had given from a larger series the value pH 5.3. If the cells be not washed after the addition of immune serum which was present in a concentration of 0.5 per cent by volume, the optimum occurs at a slightly higher figure, pH 5.5 approximately.

If a similar small volume of active normal rabbit serum be added to the cells in place of the immune serum, the optimum for agglutination occurs at the same point, pH 5.5.

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<sup>1</sup> Coulter, C. B., *J. Gen. Physiol.*, 1920-21, iii, 309.

<sup>2</sup> Coulter, C. B., *J. Gen. Physiol.*, 1920-21, iii, 513.

The euglobulin precipitates most promptly and completely from rabbit serum diluted 1:20 with distilled water at the same reaction, pH 5.5, and it is apparent that the agglutination of the cells is intimately related to the precipitation of the serum euglobulin.

The same relation is observed in the agglutination of sheep cells to which a like small amount of their own active serum has been added, as shown by the following values for the optimal point for agglutination in the presence of homologous serum: pH 5.58, 5.44, and 5.38. The euglobulin itself precipitates best from sheep serum diluted 1:20 with distilled water at approximately pH 5.5.

Guggenheimer<sup>3</sup> has made an observation which corresponds closely with this, that if defibrinated sheep blood be washed directly with isotonic saccharose solution the euglobulin of the serum is carried down with the cells and will serve as the mid-piece fraction of complement to persensitize the cells on the subsequent addition of sensitizer.

The relation mentioned is noted again when sensitized sheep cells in saccharose solution are persensitized by the addition of active normal guinea pig serum. If such serum be added in the amount of 8 per cent of the total volume to an emulsion of sensitized cells of such concentration that one unit of complement is present, the optimal point for agglutination has been found at the following electrometric values: pH 6.19, 6.35, and 6.15. Five other experiments in which the estimation was made colorimetrically gave values between pH 5.9 and 6.3. The euglobulin has been found to precipitate best from guinea pig serum diluted 1:20 with distilled water between pH 6.2 and 6.4 (electrometric).<sup>4</sup>

If the cells were persensitized at pH 6.2 and washed by allowing them to settle spontaneously from pure saccharose solution of pH 6.0 the optimal point of agglutination was noted at the following reactions (electrometric): pH 5.71, 5.79, 5.76 to 6.18, 5.38 to 5.80, 5.78, and 5.69 to 5.77. This shift toward a more acid zone runs parallel with that observed in the precipitation of guinea pig globulin which has been washed as precipitate and redissolved by bringing to pH 7.4 with NaOH. Precipitation then has its optimum between pH 5.1 and 5.7.<sup>4</sup>

<sup>3</sup> Guggenheimer, H., *Z. Immunitätsforsch. Orig.*, viii, 1910-11, 295.

<sup>4</sup> Coulter, C. B., *J. Gen. Physiol.*, 1920-21, iii, 771.

A specific reaction in the immunological sense may be supposed to take place between the sheep cells and the immune or native sensitizers of rabbit or guinea pig serum. A reaction of the same nature can hardly be thought of as occurring between sheep cells and their own serum, and yet these sera act alike as protective colloids to sheep cells since in their presence agglutination of the cells is not observed at pH 4.7; and further, they sensitize the cells to agglutination at the characteristic reactions of the serum euglobulins.

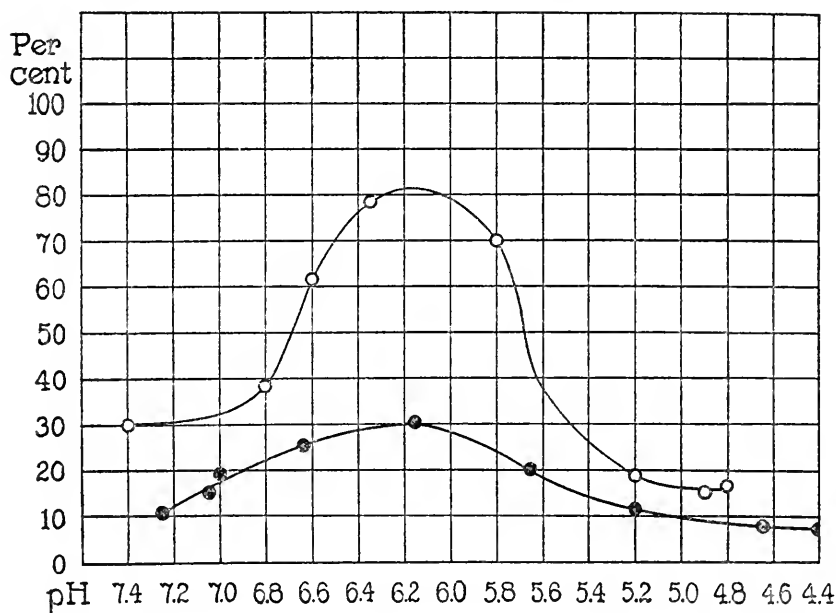


FIG. 1. Each curve represents a single experiment and gives as ordinates the percentage of the total amount of complement added which has been absorbed by sensitized cells at 4°C., with varying H ion concentrations as abscissæ.

If the cells from the tubes to which guinea pig serum has been added be sedimented and reemulsified in isotonic saline solution, it is found that they are not only persensitized, but within a certain range of reaction have combined apparently with the end-piece fraction of complement as well, since hemolysis occurs without further addition of end-piece. This is shown in the experiments recorded in the curves (Fig. 1). The phenomenon is observed regardless of whether the

cells have first been sensitized with rabbit serum or not; in the latter case the normal sensitizer of guinea pig serum, too small in amount to be recognized by the usual means, is probably united with the cells. In the experiments recorded cells were sensitized with approximately 8 units of sensitizer and added to tubes along with guinea pig serum in such amounts as to give a concentration of one unit of complement. N/10 NaOH and HCl were immediately added and the tubes kept at 4°C. for 45 minutes. The cells were then sedimented, the supernatant fluids pipetted off as completely as possible, and their pH measured electrometrically. The cell sediment in each tube was reemulsified in a constant amount of isotonic saline solution and the tubes were incubated at 37°C. The curves show the percentage of the total complement present which has been absorbed by or removed along with the cells. This percentage was plotted from the titration curve of the complement alone according to the method described by Brooks<sup>5</sup> and employed with slight modification<sup>4</sup> by the author. Five other experiments have given the same result; namely, that at the point of optimal agglutination of the persensitized cells the greatest amount of whole complement has been bound by the cells.

Guggenheimer<sup>3</sup> found that sensitized sheep cells in saccharose solution carry down with them in sedimentation the mid-piece fraction of guinea pig serum, and that the amount of mid-piece removed increases with the degree of sensitization of the cells, so that a true binding probably occurs. He could not detect, however, an absorption of the whole of complement by sensitized cells under these circumstances, even when the cells had been sensitized with 100 units. In the experiments described above in which such an absorption appears to have taken place a small amount of end-piece must have been present in the liquid phase of the sedimented cell mass, which could not have been removed by washing without disturbing the equilibrium relations between the cells and the sensitizer<sup>2</sup> and complement. According to the experience of Zinsser,<sup>6</sup> the trace of end-piece retained by the globulin sediment in the partition of complement by dialysis is sufficient to give complementary power to the globulin

<sup>5</sup> Brooks, S. C., *J. Med. Research*, 1919-20, xli, 399.

<sup>6</sup> Zinsser, H., *Infection and resistance*, New York, 1914, 180.



sediment alone. However, in the experiment recorded in the upper curve of Fig. 1, an hemolysis corresponding to an activity of 78 per cent of the total amount of complement originally present points distinctly to a true binding of end-piece by the persensitized cells.'

It is generally believed that the protective and sensitizing effects of one colloid upon another with reference to precipitation by a third substance are due to a combination of some sort between the two colloids. There is no evidence available on which to base a judgement as to the nature of the combination between the cells and the serum euglobulins considered here, whether physical or chemical. However, the fact that the presence of the sera displaces the optimum for agglutination of the cells quite sharply to the reaction characteristic of the flocculation of the euglobulin added, or of that added last and in largest amount as in the case of the guinea pig serum, suggests the occurrence of a surface combination or condensation of the serum protein upon the surface of the red cell. The conclusion is supported by the observation of Porges<sup>7</sup> that bacteria which have been treated with such an excess of immune serum that agglutination does not appear, show the salt precipitation limits of the serum euglobulin and not those of the native bacteria.

The cells in the experiments reported here were not permeable to hemoglobin since hemolysis did not appear, and as will be mentioned later were very little permeable to inorganic ions, so that a penetration of the euglobulin into the interior of the cell does not seem possible. It would be difficult to explain furthermore how the small amount of serum protein relative to the mass of cells could give its own point of optimum flocculation to a mixture of cells and serum in any other way than as a surface deposition.

The euglobulin appears to be the active protein of the serum in combining with the cells.

A phenomenon based, as far as one may judge, upon precisely the same mechanism in the combination of a protein with a surface has been observed by Loeb.<sup>8</sup> This author has found that collodion membranes always acquire the characteristics of the protein with which

<sup>7</sup> Porges, O., *Centr. Bakt., Orig.* 1906, xl, 133.

<sup>8</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 577.

they have been brought into contact, and that if such a membrane be treated with a solution of gelatin or oxyhemoglobin, for example, after the surplus protein has been washed away, the isoelectric point of the membrane is now that of the protein with which it has been treated.

This observation in connection with those reported here indicates the importance of factors which are non-specific in the serological sense in the mechanism of agglutination.

This view of the importance of the surface conditions in agglutination receives further support if the H ion concentration of the interior of the red cells be examined in relation to that of the fluid in which they are suspended. This can be done by sedimenting the cells, removing the supernatant fluid and dissolving the cell sediment in a small amount of distilled water. Experiments which are shortly to be reported in full have shown that on the acid side of pH 7.4 at least, the reaction of the interior of fresh cells in a medium of low electrolyte content is maintained at a more alkaline level than that of the fluid outside. The following figures give the reactions of the outside fluid and of the dissolved cells in an experiment in which agglutination occurred at the two most acid reactions, although not promptly:

pH outside.	pH inside.
5.92	7.28
5.83	7.26
5.61	7.18
5.44	7.03
4.08	6.97

In numerous other experiments the suspending fluid has been brought very near pH 4.8 with the appearance of immediate agglutination, and the reaction of the dissolved cells found to lie between pH 7.20 and 6.8 if the reaction be measured within 15 minutes after the addition of acid.

The ionic state of the hemoglobin must be a large factor in the electric charge carried by the cell as a whole; the value pH 4.6, in the suspending fluid, as the isoelectric point of red blood cells<sup>1</sup> appears thus to correspond under the conditions of the determination with a

reaction within the cell of pH 6.8, which is the isoelectric point of hemoglobin.<sup>9</sup>

With older cells the reaction within the cell may be at pH 6.8 when that of the outside fluid is at the same point without the appearance of a trace of agglutination. The phenomenon of agglutination appears then to be related closely to an optimal reaction in the suspending fluid and probably of the cell membrane and not to a definite reaction in the interior of the cell.

#### CONCLUSIONS.

1. The addition of blood serum displaces the optimum for agglutination of red blood cells in a salt-free medium to the reaction characteristic of flocculation of the serum euglobulin.

2. This effect is not due merely to a mechanical entanglement of the cells by the precipitating euglobulin, since at reactions at which the latter is soluble it protects the cells from the agglutination which occurs in its absence.

3. A combination of some sort appears therefore to take place between sheep cells and sheep, rabbit, and guinea pig serum euglobulin, and involves a condensation of the serum protein upon the surface of the red cell.

4. At the optimal point for agglutination of persensitized cells both mid- and end-piece of complement combine with the cells.

5. Agglutination is closely related to an optimal H ion concentration in the suspending fluid, and probably of the cell membrane, and not to a definite reaction in the interior of the cell.

<sup>9</sup> Michaelis, L., and Takahashi, O., *Biochem. Z.*, 1910, xxix, 439. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xli, 102. Michaelis, L., and Airila, Y., *Biochem. Z.*, 1921, cxviii, 144.



## THE RELATIVE TOXICITY OF THE HALIDES AND CERTAIN OTHER ANIONS.

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When iodides are administered in ordinary doses excretion takes place rapidly. Heavy doses can usually be administered before toxic symptoms develop. The same is true to an even greater extent of bromides. The bromide ion appears to be capable of replacing the chloride ion to a considerable degree in the body fluid of mammals without the production of distinct toxic symptoms. Thus Frey (1910-11) has shown that while administration of sodium bromide (to rabbits) leads at first to increased excretion of sodium chloride, if the relative amounts in the diet are kept constant bromide and chloride excretion become parallel; while an animal kept for some time on a deficient chloride diet, and then fed bromide, tends to retain bromide equally with chloride.

There is evidence that some at any rate of the toxic symptoms following heavy administration of bromide are actually due to too great a depletion of chloride from the body, and not to the bromide ion (*cf.* Wolff and Opp, 1912).

After bromide administration hydrobromic acid occurs in the gastric juice (Wolff and Opp).

Similarly Herzfeld and Heimann (1911) have shown that after administration of iodide the excretion of chlorides and iodides is in inverse ratio. Hydriodic acid occurs in the gastric juice after iodide administration, and iodide is found in saliva, tears, perspiration, milk, sebum, and the secretion of the nasal mucous membrane (Cushny, 1918).

It is to be noted that Hale and Fishman (1908) state that bromide is excreted more slowly than corresponding doses of iodide.

According to Biedermann (1921), animal and vegetable diastases consist of a thermostable, organic component, by itself inactive, and a thermostable coenzyme. Numerous inorganic salts can act as the coenzyme, mainly through the action of their anions. The sodium salts in order of greatest effect are the chloride, bromide, nitrate, iodide, and sulphate.

The bromide ion distributes itself between blood corpuscles and plasma in a ratio (1:3.1) similar to that for the corresponding distribution of chloride ions (1:2.1) (Wiechmann, 1921).

It is evident that bromides and iodides can to a certain extent replace chloride in the circulating and tissue fluids and secretions of the body with apparently the same functional effect, and without producing definite toxic symptoms. This research, carried out during the winters of 1919-20 and 1920-21, is directed toward the solution of the problem of to what extent such non-toxic replacement can take place.

In view of the fact that the iodine of sea water is present chiefly as iodate (Sonstadt, 1872, and Winkler, 1916) a comparative study of iodate has been included.

As a basis of comparison we have measured the survival times of frog heart and muscle-nerve preparations immersed in modified Locke solutions containing mixtures of the ions studied.

While much work has been carried out in determining the relative effects of various series of cations on the skeletal and cardiac muscles of the frog, less has been done with series of anions.

Loeb (1909) has shown that the relative effectiveness of halide ions in producing rhythmic contractions (a toxic effect) in muscle is given by the order,  $F > I > Br > Cl$ , the minimal concentrations being for NaF,  $m/64$  to  $m/96$ ; for NaI,  $m/32$ ; for NaBr,  $m/16$  to  $m/32$ ; and for NaCl,  $m/16$ . This is connected, as Loeb points out, with the rapidity of passage into the muscle. He has recently shown (1919-20) that the same order holds for the influence of these ions on the rate of diffusion of water through collodion membranes.

Kruse (1919) has shown that a bromide-Ringer or Locke solution is inadequate to maintain rhythmicity in a perfused isolated mammalian heart for as long a time as a similar chloride solution, but claims that in the frog heart the bromide-Ringer solution is at least as effi-

cient as a chloride-Ringer solution. Macht and Hooker (1918) have shown that perfusion of the medulla of a mammal by a bromide solution stimulates the respiratory and cardio-inhibitory centers. Kruse considers therefore that the bromides are more or less irritating to most tissues of the body, but states that the central nervous system of the frog is not depressed by sodium bromide.

Stiles (1901) showed that isolated strips of frog esophagus are maintained in rhythm by bromide-Ringer solution. Prochnow (1911) has shown that the sodium halides increase the tonus of the uterus and the smooth muscle of arteries in the order, NaF, NaI, NaBr, the excitability being increased. Kruse found that bromides stimulated smooth muscle (intestine of dog or cat) slightly under normal conditions, and markedly when arrhythmic or fatigued.

Greisheimer (1919) found that M/8 sodium bromide is more toxic than M/8 sodium chloride as judged by the effect on the motor nerve of the frog.

Finckh (1921) has tested the effects of sodium halides on the isolated frog heart. In bromide-Ringer solution the heart continues beating for a long time, whence Finckh concludes that the chloride ion plays no peculiar rôle in this connection. When iodide-Ringer solution is substituted, harmful effects are observed which are attributed to the formation of elementary iodine. Nitrate-Ringer solution gives a similar result; here the toxic effect is explained by the possible formation of  $\text{NO}_2$  ions.

### *Experimental.*

We have determined the survival periods of frog heart and gastrocnemius-sciatic preparations (*Rana pipiens*) immersed in modified Locke solutions in which varying amounts of sodium chloride were replaced by the corresponding molecular concentrations of the sodium salts of the anions studied, fluoride, bromide, iodide, chlorate, iodate, and nitrate (Table I). In each set of experiments the solutions were made up with freshly distilled water, and the preparations, dissected as quickly as possible, were immersed in them in shallow vessels, so that the oxygen supply could be regarded as sufficient. The volume of solution, compared to volume of tissue, was large. From time to time the preparations were observed and tested. The times

were noted at which the hearts ceased to respond to electrical stimulation (the times at which normal heart beat ceased were irregular), and the muscles ceased to respond to such stimulation applied (a) directly, and (b) through their nerves. In all cases it was found that nerve tissue died shortly before the death of the corresponding muscle.

TABLE I.  
*Composition of Modified Locke Solutions.*

	M/7.8 NaF	M/7.8 NaCl	M/7.8 NaBr	M/7.8 NaI	M/7.8 NaClO <sub>2</sub>	M/7.8 NaIO <sub>2</sub>	M/7.8 NaNO <sub>2</sub>
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
NaF.....	5.38						
NaCl.....		7.50					
NaBr.....			13.20				
NaI.....				19.23			
NaClO <sub>2</sub> .....					13.65		
NaIO <sub>2</sub> .....						26.0	
NaNO <sub>2</sub> .....							10.9
NaHCO <sub>3</sub> .....	0.10	0.10	0.10	0.10	0.10	0.10	0.10
KCl.....	0.075	0.075	0.075	0.075	0.075	0.075	0.075
CaCl <sub>2</sub> .....	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Glucose.....	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Water to.....1,000 cc.							

It was observed very early in the experiments that a difference of a few degrees of temperature had a marked effect on the duration of life of the muscle-nerve preparations, in whatever solutions they were immersed. This is in agreement with previous observations of Cameron and Brownlee (1915-16) (Table II), while Gunn and Underhill (1914-15) have shown that temperature produces a similar effect in surviving mammalian tissue (intestine). Utilizing the fairly stable temperature conditions of the city water supply through the long winter here, it was possible to obtain observations throughout which the temperature remained fairly constant at 5°C. The shallow beakers containing the preparations were immersed in a large sink through which water from the city main flowed constant, and in most of the experiments the solution temperatures were thus kept constant to 5° ± 1°C. By selecting suitable times experiments at



TABLE II.  
*The Effect of Temperature.*

Experimental Series No.	Number of frogs used.	Temperature.	Survival times.								
			Heart.			Muscle.			Nerve.		
			Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.
100 per cent M/7.8 NaCl solutions.											
2	6	5	48.4	45.8	47.2	173.8	167.7	171.9	173.0	166.9	170.0
7	4	5	58.4	54.0	56.7	222.7	211.2	218.5	210.7	202.2	207.9
10	6	5	58.8	57.6	58.2	230.7	227.2	228.9	228.5	226.0	227.7
1	7	5-8	57.3	49.0	52.3	172.8	167.2	169.6	171.8	162.5	168.9
3	6	5-8	46.1	43.9	44.9	169.9	162.7	165.1	167.5	161.3	165.0
5	4	15-16	3.5	3.1	3.3	49.0	47.5	48.3	48.3	47.0	47.4
6	6	25	2.9	2.2	2.5	8.4	7.6	8.0	8.1	7.2	7.7
Cameron and Brownlee.	6	29				Less than 6.					
	3	32	Less than 1.								
	6	36				Less than 2.					
	6	39				Less than 1.					
100 per cent M/7.8 NaBr solutions.											
1	7	5-8	33.4	30.4	32.3	123.9	121.8	122.1	122.7	120.9	121.8
5	4	15-16	2.4	2.2	2.2	35.0	34.3	34.8	34.5	34.0	34.2
6	6	25	1.7	1.6	1.7	7.1	6.8	6.7	6.8	6.2	6.4
100 per cent M/7.8 NaI solutions.											
1	7	5-8	8.1	7.5	7.6	46.4	44.5	45.7	45.5	43.1	44.4
5	4	15-16	0.87	0.77	0.83	11.4	10.3	10.8	10.7	10.2	10.4
6	6	25	1.12	0.75	0.97	4.1	3.3	3.7	3.8	3.2	3.5
100 per cent M/7.8 NaF solutions.											
1	7	5-8	0.18	0.12	0.13	1.67	1.25	1.43	1.33	0.95	1.25
5	4	15-16	0.15	0.13	0.14	0.65	0.52	0.57	0.57	0.47	0.52
6	6	25	0.13	0.10	0.12	0.48	0.37	0.42	0.45	0.32	0.37
100 per cent M/7.8 NaClO <sub>3</sub> .											
4	6	5	20.0	18.2	19.3	78.5	74.7	76.3	77.3	73.2	75.2
5	4	15-16	1.8	1.6	1.7	14.9	14.2	14.7	14.2	13.5	14.1
6	6	25	1.5	1.1	1.3	4.9	4.2	4.5	4.5	3.8	4.2
100 per cent M/7.8 NaNO <sub>3</sub> .											
4	6	5	14.6	12.7	13.7	61.8	57.8	60.3	60.5	56.7	59.2
5	4	15-16	2.1	1.9	2.0	18.9	18.2	18.7	18.7	18.0	18.3
6	6	25	2.0	1.7	1.8	5.0	4.7	4.8	4.7	4.3	4.5

TABLE III.  
*Effect of Halide Mixtures.*

Experimental Series No.	Number of frogs used.	M/7.8 NaX	M/7.8 NaCl	Temperature.	Survival times.								
					Heart.			Muscle.			Nerve.		
					Maxi- mum.	Mini- mum.	Mean.	Maxi- mum.	Mini- mum.	Mean.	Maxi- mum.	Mini- mum.	Mean.
Experiments with NaBr.													
		<i>per cent</i>	<i>per cent</i>	°C.	<i>hrs.</i>	<i>hrs.</i>	<i>hrs.</i>	<i>hrs.</i>	<i>hrs.</i>	<i>hrs.</i>	<i>hrs.</i>	<i>hrs.</i>	<i>hrs.</i>
1	7	100	0	5-8	33.4	30.4	32.3	123.9	121.8	122.1	122.7	120.9	121.8
2	6	50	50	5	36.9	34.6	35.5	143.7	139.6	142.4	142.8	138.4	121.2
3	6	25	75	5-8	46.1	43.8	44.9	169.9	162.7	165.1	167.5	161.3	165.0
7	4	12	88	5	46.6	43.7	45.3	194.7	191.2	193.0	186.2	182.7	184.4
8	4	5	95	5	51.6	50.0	50.7	202.5	197.0	200.1	192.6	190.7	191.4
7	4	0	100	5	58.4	54.0	56.7	222.7	211.1	218.5	210.7	202.2	207.9
Experiments with NaI.													
1	7	100	0	5-8	8.1	7.5	7.6	46.4	44.5	45.7	45.5	43.1	44.4
2	6	50	50	5	12.3	11.5	12.0	64.7	60.9	62.9	63.7	60.2	61.8
3	6	25	75	5-8	15.8	14.6	15.1	74.0	67.8	70.5	72.7	66.5	69.0
7	4	12	88	5	29.2	27.3	28.2	123.2	120.7	121.8	112.3	111.2	111.7
8	4	5	95	5	32.7	31.2	32.2	131.6	130.7	131.2	122.6	120.7	121.5
9	7	2.5	97.5	5	42.7	40.9	42.2	171.0	168.7	169.7	162.7	159.6	160.7
10	6	5	95	5	34.2	34.0	34.1	131.6	130.3	130.7	131.2	129.9	130.4
10	6	4	96	5	38.2	37.4	38.0	140.0	139.0	139.5	139.7	138.8	139.5
10	6	3	97	5	41.7	41.0	41.5	154.0	153.0	153.7	153.7	152.7	153.3
10	6	2	98	5	47.5	46.2	46.8	189.3	188.7	188.7	188.7	188.2	188.3
10	6	1	99	5	52.5	52.0	52.3	213.5	212.3	212.6	212.7	211.7	212.2
10	6	0	100	5	58.8	57.6	58.2	230.7	227.2	228.9	228.5	226.0	227.7
Experiments with NaF.													
1	7	100	0	5-8	0.18	0.12	0.13	1.67	1.25	1.43	1.33	0.95	1.25
2	6	50	50	5	1.9	1.2	1.5	6.3	4.7	5.3	5.8	4.1	4.8
3	6	25	75	5-8	4.6	2.7	3.4	8.4	6.3	6.9	7.4	5.8	6.4
7	4	12	88	5	12.4	11.9	12.0	22.7	21.7	22.7	20.4	19.4	20.1
8	4	5	95	5	25.0	24.7	24.8	49.7	48.7	49.1	40.7	39.0	40.0
7	4	0	100	5	58.4	54.0	56.7	222.7	211.2	218.5	210.7	202.2	207.9
Experiments with NaClO <sub>3</sub> .													
4	6	100	0	5	20.0	18.2	19.3	78.5	74.7	76.3	77.3	73.3	75.2
2	6	50	50	5	21.7	20.4	21.0	82.5	79.0	81.1	81.9	78.6	80.1
3	6	25	75	5-8	25.4	23.5	24.5	89.8	82.8	86.8	87.6	81.7	85.1
7	4	12	88	5	33.0	32.0	32.5	149.2	146.7	147.7	139.7	136.8	138.6
8	4	5	95	5	42.1	40.8	41.3	161.0	159.7	160.5	151.6	149.8	150.7
7	4	0	100	5	58.4	54.0	56.7	222.7	211.2	218.5	210.7	202.2	207.9

TABLE III—*Continued.*

Experimental Series No.	Number of frogs used.	M/7.8 NaX	M/7.8 NaCl	Temperature.	Survival times.								
					Heart.			Muscle.			Nerve.		
					Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.
Experiments with NaIO <sub>3</sub> .													
		per cent	per cent	°C.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.
10	6	10	90	5	24.7	24.0	24.3	96.6	93.4	95.0	95.0	92.6	94.0
10	6	5	95	5	31.6	30.0	30.7	121.8	120.0	121.5	121.5	119.7	121.2
10	6	4	96	5	34.0	33.0	33.2	134.0	133.4	133.7	133.6	133.1	133.4
10	6	3	97	5	37.7	37.2	37.5	144.7	143.8	144.3	144.2	143.5	143.8
10	6	2	98	5	43.0	42.0	42.5	158.8	157.6	157.8	158.4	157.0	157.3
10	6	1	99	5	49.7	49.2	49.5	169.7	167.0	168.6	168.4	167.2	168.2
10	6	0.5	99.5	5	54.7	53.6	54.5	179.0	178.4	178.7	178.2	177.6	177.7
10	6	0.1	99.9	5	56.7	56.3	56.7	218.4	217.2	217.7	217.8	216.8	217.3
10	6	0	100	5	58.8	57.6	58.2	230.7	227.2	228.9	228.5	226.0	227.7
Experiments with NaNO <sub>3</sub> .													
4	6	100	0	5	14.6	12.7	13.7	61.8	57.8	60.3	60.5	56.7	59.2
2	6	50	50	5	19.4	17.4	18.2	71.2	65.7	68.6	69.4	64.7	67.3
3	6	25	75	5-8	23.6	19.7	21.4	73.7	70.9	72.3	72.0	69.4	70.6
7	4	12	88	5	29.6	28.2	28.7	136.3	134.0	134.6	125.8	123.1	125.2
8	4	5	95	5	35.7	35.0	35.3	140.7	138.7	139.5	131.2	130.0	130.5
9	7	2.5	97.5	5	38.9	36.7	37.6	150.7	147.5	149.4	140.7	138.1	139.7
10	6	5	95	5	38.5	37.7	38.0	140.0	139.0	139.7	139.7	138.7	139.4
10	6	4	96	5	40.7	39.9	40.5	151.4	150.0	150.5	151.2	149.7	151.2
10	6	3	97	5	42.5	41.5	42.2	160.4	160.0	160.1	160.2	159.7	159.7
10	6	2	98	5	43.6	43.2	43.5	172.2	171.1	171.5	171.7	170.7	171.3
10	6	1	99	5	49.2	48.9	49.1	187.2	186.3	186.9	186.7	185.7	186.6
10	6	0.5	99.5	5	55.1	54.8	55.0	200.2	199.4	199.9	199.7	198.9	199.6
10	6	0.1	99.9	5	56.0	55.1	55.7	213.7	212.4	212.9	213.3	212.1	212.5
10	6	0	100	5	58.8	57.6	58.2	230.7	227.2	228.9	228.5	226.0	227.7

other temperatures were made, with approximately the same degree of variation. In this way the effect of temperature has been ascertained fairly accurately. These temperature results are shown in Table II.

The modified Locke solutions employed (Table I) were equimolecular. Mixtures in known proportions gave solutions in which defi-

nite percentages of sodium chloride were replaced by the corresponding molecular amounts of the other anions. The actual molecular concentration of halide was therefore always  $M/7.8$ . With the degree of dilution employed, differences in degree of ionization can be disregarded. Table III shows the effects of such mixtures, for each of which the molecular percentage of the anion studied is indicated as "NaX."

The chemicals used were of the following degree of purity: sodium chloride, sodium hydrogen carbonate, potassium chloride, and calcium chloride, Kahlbaum, "zur Analyse" (pre-war manufacture); sodium fluoride, glucose, Merck's pure; sodium bromide, sodium iodide, sodium chlorate, sodium iodate, and sodium nitrate, Merck's U.S.P.

In all cases the animals were weighed before the experiment. Assuming that the weights of the tissues used were proportional to total body weight, there was no proportionality between weight of tissue and duration of life. The extreme weight limits in all experiments were 30 and 67 gm. In each experiment the extreme difference was much smaller. Almost all the animals were male.

#### DISCUSSION.

Our results show a fair degree of constancy, as exhibited both by the figures for the same conditions in different experimental series (the experiments in any particular series were commenced at approximately the same time and therefore those in one series are somewhat more accurately comparable) and the extreme variations obtained under any one set of conditions. The actual differences observed in the three different experiments with chloride-Locke solution at  $5^{\circ}\text{C}$ . are probably in part referable to different temperature fluctuations within the slight limits mentioned ( $5 \pm 1^{\circ}\text{C}$ ). The maximum survival time of frog muscle in chloride-Locke solution was 230.7 hours; of frog nerve, 228.5 hours; and of frog heart muscle, 58.4 hours. These figures appear to be somewhat greater than any previously recorded, the difference being almost certainly due to temperature.

The period of survival decreases with increase of temperature in all solutions. The results for frog muscle are shown in Fig. 1. Those for heart and nerve give similar curves. (The mean temperature for

Experimental Series 1 and 3 has been taken as 6.5.) The halide curves for muscle are logarithmic; those for nitrate and chlorate do not show this relationship. The decrease is probably due to the combined effect of temperature *per se* (especially evident at higher temperatures and due presumably to an acceleration of all metabolic processes in the tissue) and of temperature in increasing the toxic effect of the foreign ion.

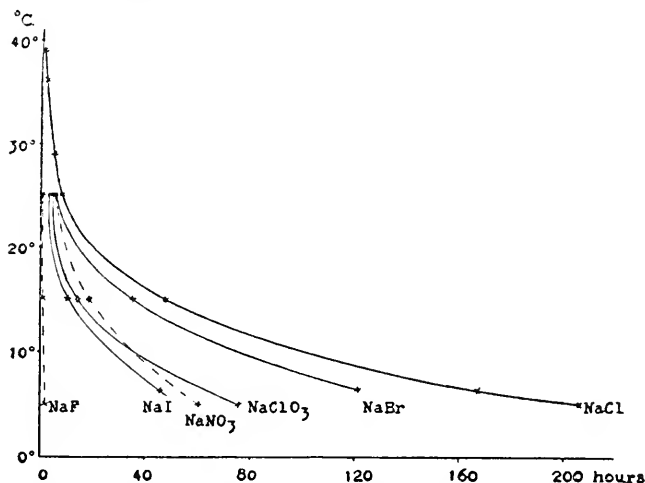


FIG. 1. Effect of temperature on the survival period of frog muscle immersed in various Locke solutions.

Introduction of any foreign ion into the Locke solution, under constant temperature conditions, decreases the survival period. This must be regarded as due to toxicity of the foreign ion. The greatest relative toxic effect appears to be produced by an initial slight replacement of chloride ions. Further replacement does not produce such a relatively great effect. This is shown by the curves for frog muscle in Fig. 2. Nerve and heart data yield similar curves.

It is therefore apparent that even small traces of such foreign ions (including bromide and iodide) are distinctly, though but slightly toxic. This is not in agreement with Finckh's conclusions, but is in general agreement with Kruse's results. Since, in solutions containing only a small percentage of replacements by the foreign ions, the survival period is in most cases at least several days, it seems un-

likely that the controlling factor is the relative permeability of the cell membrane to these ions. The important factor is probably a direct chemical action of the foreign ion on the cell protoplasm or some part of it.

Finckh has shown that very similar results are obtained with iodide and nitrate-Ringer solutions. Our results are in agreement, but it seems to us, on chemical grounds, since this parallel toxicity

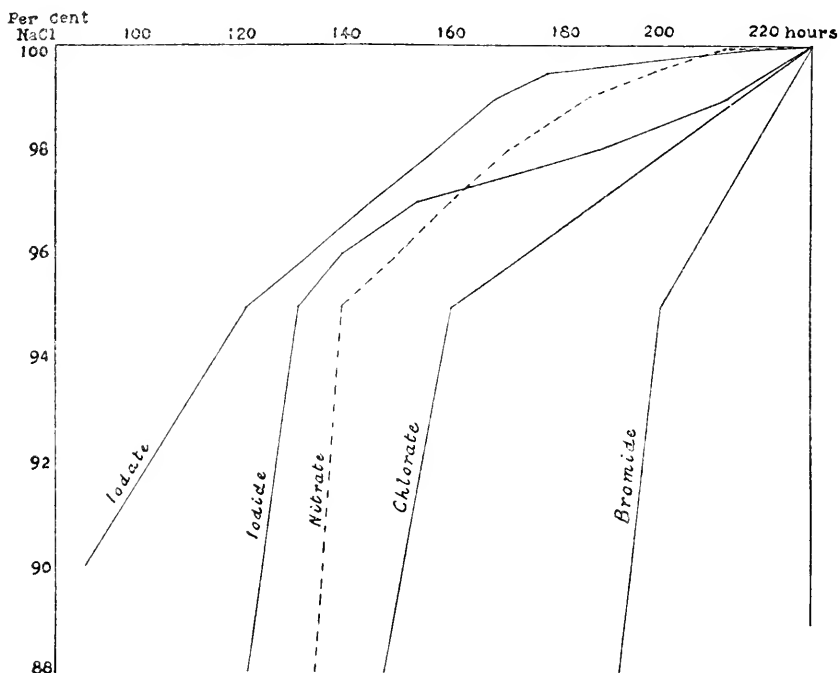


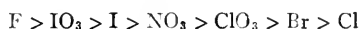
FIG. 2. Effect of substitution of chloride in Locke solutions by other anions on the survival period of frog muscle.

persists in solutions in which the I and  $\text{NO}_3$  concentrations are very slight, that it is extremely unlikely that Finckh's conclusion is correct that the toxicity can be traceable to the formation of elementary iodine and of  $\text{NO}_2$  ions.

Our results show (Fig. 2) a distinct difference of action in solutions in which 2 and 3 per cent of chloride ion is replaced by iodide and nitrate. For the greater replacement iodide is more toxic than nitrate;

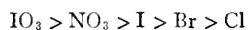
for the lesser iodide is less toxic. This sudden decrease in degree of toxicity with greater dilution seems to be in agreement with the non-observance of distinct toxic effects after administration of small doses of iodide to mammals. There would appear to be a similar abnormal decrease for still smaller iodate concentrations, as contrasted with nitrate-chloride mixtures (Fig. 2). Nevertheless, even very slight traces of any of the foreign ions that we have tested exhibit a distinct toxicity, when compared with pure chloride-Locke solution.

For Locke solutions in which more than 5 per cent of chloride is replaced by the corresponding molecular concentration of the foreign ion, the toxicity is in descending order



This is in agreement with the effectiveness in producing rhythmic contractions in muscle (Loeb); the inverse order is that found by Biedermann for effectiveness as coenzymes of amylases.

When less than 2 per cent of the chloride is replaced the order is



These results can almost certainly be considered to hold for body fluids circulating normally and containing these foreign ions.

The much greater relative toxicity of fluoride solution under all conditions is in agreement with the marked biochemical differences of fluoride and the other halide ions.

M/7.8 chlorate-Locke solution produced a regular series of rhythmic contractions (about 35 per minute) in muscle. M/7.8 iodide-Locke solution produced a similar effect; the rate was somewhat faster. Fluoride produced a continuous fibrillation. The chlorate and iodide effects were not observed with muscle of curarized frogs, though the fluoride fibrillation persisted. No such effects were observed with the other anions studied.

This research forms part of a series relating to the biochemistry of iodine. Part of the expenses were defrayed by a grant from The Chemical Society (of London) to whom we tender our thanks.

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## SOME EFFECTS OF RADIUM RADIATIONS ON WHITE MICE.

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It is well known that radium rays, as well as x-rays, are capable of affecting the living cell. Bergonié and Tribondeau<sup>1</sup> from extensive experimental studies made the generalization<sup>2,3</sup> that: "Immature cells and cells in an active state of division are more sensitive to the x-rays than are cells which have already acquired their fixed adult, morphological or physiological characters."<sup>2</sup> Subsequent observations have resulted in the extension of this generalization to the effect of radium radiations as well. For this reason the mice of the experiments described in the present paper were exposed to radium rays soon after birth and during the period of rapid growth.

### *Experimental.*

The body weight of mice at birth and their subsequent rate of growth are influenced by many factors. It is essential, therefore, that control experiments be carried out with animals as nearly as possible like those treated; and that external conditions be identical for both groups during the period of observation. The age, physical condition, and body weight of the mother, the length of the gestation period, the size of the litter, and its position in the litter series affect the initial weight of mice and their growth. Accordingly, a litter of young was divided into two nearly equal groups of both sexes; one to be exposed to radiation and the other to be used as control. The method devised

<sup>1</sup> Bergonié, J., and Tribondeau, L., *Compt. rend. Acad.*, 1906, cxliii, 983

<sup>2</sup> Colwell, H. A., and Russ, S., Radium, x-rays, and the living cell, London 1915, 253.

<sup>3</sup> Bécélère, M., Cottentot, P., and Laborde, S., *Radiologie and radiumthérapie* Paris 1921 223.

by Jackson<sup>4</sup> for albino rats was used to determine the sex of the new born mice. Any underdeveloped animals were discarded, but otherwise the litter was divided into the two groups at random. In this way individual inequalities would be divided fairly evenly between the two groups. It was found, in fact, that the average weight of the mice of one group from day to day was very nearly the same as for the other group, when neither was treated with radium.

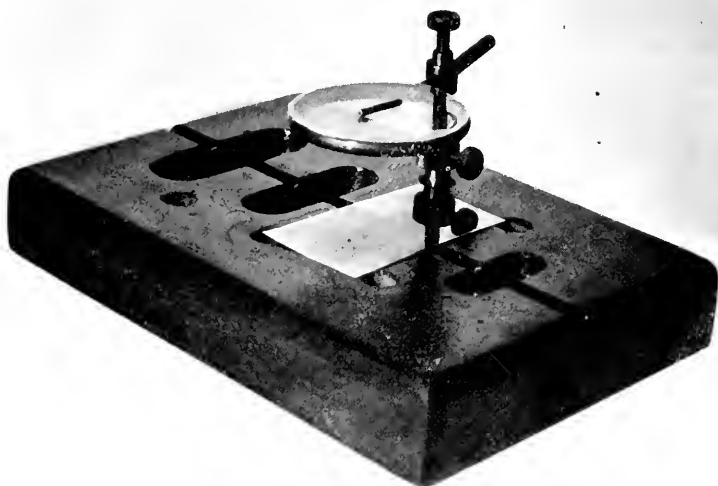


FIG. 1.

The apparatus of Fig. 1 was used to irradiate the mice, one at a time. The new born animal was placed in a cavity slightly larger than itself and was covered with a very thin sheet of mica. In this manner the mouse remained in a fixed position during the treatment, with its back touching the mica. A capillary glass tube 10 to 14 mm. in length, and about 0.5 mm. in external diameter, containing radium emanation, was placed on another sheet of mica which was rigidly supported at a distance of 2 cm. from the animal. The holder was

<sup>4</sup> Jackson, C. M., *Biol. Bull.*, 1912, xxiii, 171.

TABLE I.  
*Experimental conditions, and doses of radiation.*

Experiment No.	No. of mice radiated.		No. of controls.		Part of body radiated.	Distance between animal and tube.	Duration of daily exposure.	Strength of emanation tube used.											Total dose.		
	Male.	Female.	Male.	Female.				1st day.	2nd day.	3rd day.	4th day.	5th day.	6th day.	7th day.	8th day.	9th day.	10th day.	11th day.		12th day.	
								cm.	min.												milli- curie hours
1	1		2	2	2	Back.	2	5	4.3	6.0	5.2	4.0	3.8							1.9	
2	2		2	2	2	"	2	5	5.4	4.5	6.7	6.7	5.6							2.4	
3	2		2	2	1	"	2	5	5.0	6.0	5.0	5.0	4.5							4.9	
4	1		2	1	1	Head.	2	5	10.5	9.0	7.6	6.4	5.4	8.0	6.7	8.0	6.9	7.5	6.3	6.8	
5	4		2	2	3	Back.	2	5	14.5	12.2	14.2	11.0	11.0	11.0	10.5	11.0	10.0	10.0	9.0	11.5	
6	1		1	1	3	Head.	2	5	27	23	29	30	32	33	31	26	32			21.9	
7	1		3	1	3	"	2	5	30	33	28	30	33	31	28	30	28	27	23	26.8	
8	2		2	1	1	Back.	2	5	48	48	48	53	45	38	54	45				31.6	
9	2		2	1	2	"	2	5	65	40	34	29	50	50	50	62	58			36.5	

adjusted so that the tube was either directly over the central dorsal region or over the head of the animal. The time of exposure was always 5 minutes. The amounts of emanation employed and the number of treatments given on successive days are shown in Table I.

To identify the controls from the radiated mice in the same litter a small piece of tail was clipped off from the specimens of one group. The mice of each group were weighed together for the first time within 12 hours of the time of birth, and then every 24 hours until the 21st day, when the young were suckled. From this time on the mice were weighed individually about once a week. For any group the average weight for one mouse was calculated. A careful record was kept of the physical condition, body weight, and hair development for the different mice; also the time when they opened their eyes or reached sexual maturity, and the duration of life.

The biological effect of radium radiations is related to the amount of radiant energy absorbed by the tissue. This depends on various factors, of which the quality of radiation used and the distance of application are the most important ones. At the present time there is no satisfactory unit in which to express the dose of radiation administered. In these experiments radium emanation enclosed in capillary glass tubes of a definite size was used as the source. The quality of the radiation employed, mainly  $\beta$ -rays, therefore, was always the same, and the animal was placed at a distance of 2 cm. from the emanation tube for every treatment. Under these conditions the dose depends only on the amount of emanation in the tube and the duration of the application. The former is given in millicuries and the latter can be expressed in hours; the product of the two (millicurie hours) in this case may be taken to represent the relative doses. It should be noted in this connection that in each case the millicurie hour dose appearing in Table I is the total administered during the whole period of treatment in fractional daily doses.

The typical effects of different doses are shown graphically in Fig. 2. Here the body weights of the radiated mice and their respective controls are given for a period of 8 weeks, this being the time during which the maximum radium reaction is likely to occur. It will be seen that a dose of 2.4 millicurie hours, fractionally applied, in the manner previously described, accelerated the growth of the mice.

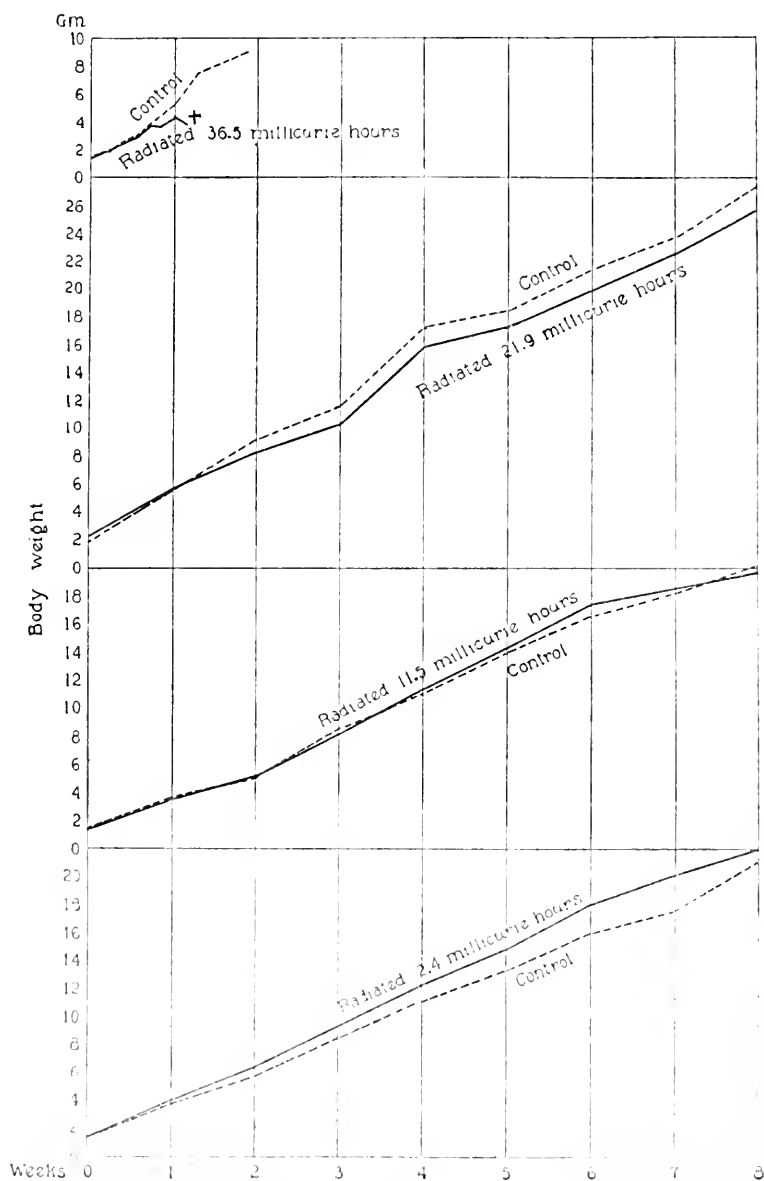


FIG. 2. Charts showing effect of radium radiation on the growth of mice. Source of radiation, one tube of radium emanation, 14 mm. long and 0.5 mm. in diameter. Distance of source, 2 cm. No metallic filter. Divided doses are shown in Table I.

The weight of the radiated mice remained distinctly larger than that of the controls until about the 27th week. After this time the average weight of the two groups was substantially the same. A dose of 11.5 millicurie hours had practically no effect on the body weight of the mice. A dose of 21.9 millicurie hours, however, had a marked influence on the growth of the radiated mice. They remained distinctly smaller than the controls for several months, but finally (in the 45th week) their average weight was the same as that of the control group. The mice which received a dose of 31.6 millicurie hours were affected very seriously by the radiation. Their growth was greatly retarded and they died on the 12th day. The effect of a dose of 36.5 millicurie hours was even more marked, and the mice died on the 9th day.

The results just described may be summarized as follows: (a) Sufficiently small doses of radiation accelerate the growth of suckling white mice. (b) A larger dose of the proper value will have no influence on the body growth of mice. (c) A still larger dose, up to a certain limit, will retard growth, but the animals will eventually attain normal size. (d) Still larger doses cause premature death. Similar results have been obtained before in experiments on seeds and plants,<sup>5,6</sup> also on lower forms of animal life exposed to x-rays.<sup>7,8</sup> From these it is commonly assumed that the action of radiation on the living cell follows the same general law<sup>9</sup> which governs the action of all anesthetics, as well as chemical, mechanical, and electrical stimulants; that is, if some form of energy is gradually brought to bear on the cells, at first they may be stimulated to greater activity, then their normal function may be arrested, and finally they may be destroyed.

As a result of the mode of application of the radium emanation adopted in these experiments, the upper part of the body of the animals was exposed to an intense radiation. The lower part received much less radiation, not only because it was farther away from the source, but also because the overlying layers of tissue absorbed most of the

<sup>5</sup> Gager, C. S., *Memoirs*, New York Botanical Gardens, 1908, iv.

<sup>6</sup> Molisch, H. C., *Sitzungsb. k. Akad. Wissensch. Math-naturw.*, Wien, 1912, cxxi, 121.

<sup>7</sup> Davey, W. P., *J. Exp. Zool.*, 1917, xxii, 573.

<sup>8</sup> X-ray Studies, General Electric Company, Schenectady, 1919, 255, 267.

<sup>9</sup> Christen, Th., *Strahlentherapie*, 1919, lx, 590.

$\beta$ -rays. The emanation tube was placed parallel to the length of the animal to obtain a more even distribution of the radiation. Nevertheless it should be expected that the skin directly under the tube would receive more radiation, especially on account of the curvature of the body. Accordingly, when the dose was sufficiently large, marked local effects were produced on the backs or heads of the animals. The results may be summarized as follows: There were no visible changes in the skin and the hair growth of mice exposed to doses of 1.9, 2.4, and 4.7 millicurie hours. A dose of 6.8 millicurie hours retarded the development of lanugo hair directly opposite the emanation tube, and produced a skin erythema. The growth of hair, however, became normal on the 15th day after birth. Larger doses of radiation produced more pronounced effects. The mice irradiated with 11.5 millicurie hours had no hair on their backs on the 8th day. The hairless area was completely filled with new hair on the 14th day, but its retarded growth could be noticed up to the 30th day. The animals exposed to 21.9 millicurie hours showed wrinkled and reddened scalps on the 10th day. The radium reaction on the skin became worse up to the 15th day, when it was at its height. The skin was dry, scaly, wrinkled, and hemorrhagic. The eyes were inflamed and almost closed; the ears red, swollen, and underdeveloped. The healing process began at this time and progressed slowly. The effects from 26.8 millicurie hours were similar to these but more severe. On the 13th day the mice were unable to run normally, but could get about only with great difficulty. However, the animals gradually regained their health and vigor. A narrow strip of hairless skin, over which the emanation tube had been applied at a distance of 2 cm. was still visible at the end of the 616 day. Doses of 31.6 and 36.5 millicurie hours caused the mice to die on the 12th and 9th days respectively. Their hind legs were completely paralyzed the day before death. The viscera were very much undersized, and the brains hemorrhagic and congested.

The age at which the normal mice open their eyes is variable, but generally it is 13 days. Among mice of the same litter, however, there is little irregularity. Female mice often open their eyes a few hours earlier than the males. A record was kept of the age of the individual mice at the time they opened their eyes, and the averages,

for each group of controls and radiated mice, are given in Table II. It will be seen that doses below 11.5 millicurie hours had no apparent effect on the time when the radiated mice opened their eyes. But the mice receiving larger doses of radiation opened their eyes from 12 to 24 hours earlier than their respective controls. Similar effects were

TABLE II.  
*The Effect of Radium Radiations on Opening of Eyes in White Mice.*

Experiment No.	Group.	No. of mice radiated.		Average age at which eyes opened.
		Males.	Females.	
				<i>days.</i>
1	Controls.	2	2	13.1
	1.9 millicurie hours.	1	2	13.0
2	Controls.	2	2	13.9
	2.4 millicurie hours.	2	2	14.0
3	Controls.	2	1	13.2
	4.9 millicurie hours.	2	2	13.1
4	Controls.	1	1	12.0
	6.8 millicurie hours.	1	2	12.0
5	Controls.	2	3	13.7
	11.5 millicurie hours.	4	2	13.2
6	Controls.	1	3	13.0
	21.9 millicurie hours.	1	1	12.0
7	Controls.	1	3	12.9
	26.8 millicurie hours.	1	3	12.2

noted by Tribondeau and Belley,<sup>10</sup> who x-rayed the eyes of young kittens and found that the radiated eyes opened always some hours before the controls.

Many experimenters have noted that radium and x-rays can bring about sterility in animals. The literature is abundant,<sup>11</sup> but in many

<sup>10</sup> Tribondeau, L., and Belley, G., *Arch. Med.*, 1907, xv, 907.

<sup>11</sup> Albers-Schönberg, *Münch. med. Woch.* 1903, I, 1859. Friebe, *Münch. med. Woch.*, 1903, I, 2295. Philipp, *Fortschr. Geb. Röntgenstrahlen*, 1904, viii, 114. Halberstädter, L., *Berl. klin. Woch.*, 1905, xlii, 64. Bergonié, J., Tri-



cases the doses of radiation which caused sterility either are not given at all or they are given incompletely. The marked response to radiation which the reproductive cells exhibit can be explained by Bergonié and Tribondeau's generalization, already referred to. In the experiments of this paper, the effect of radiation on the reproductivity of the mice was also investigated. For this purpose the radiated animals were mated with normal mice about the 35th day after birth. One radiated male was mated with two normal females of about the same age. The animals were kept in the same cage until both females had produced at least three litters of young. Two radiated females were mated with two normal males, and they were not separated until the females had reached the age at which they normally cease to breed. In general, the young were separated from the mother soon after birth. In the case of the mice of Experiments 1 and 4 the young of one litter in either experiment were allowed to remain with their mother until weaned. These mice and succeeding generations were kept under observation to determine whether radiation affected the offspring of the radiated mice.

The results of the breeding tests were as follows: All the males of Experiments 1 to 7, receiving doses of 1.9 to 26.8 millicurie hours, were fertile. (Those of Experiments 8 and 9 succumbed to doses of radiation larger than 26.8 millicurie hours before they reached sexual maturity.) On the other hand, with the exception of the mice of Experiments 1 to 4, all the radiated females were rendered permanently sterile. Those of Experiments 1 and 4 were fertile. This shows that a dose of 1.9 millicurie hours (Experiment 1) applied as already explained (Table I) was not sufficient to sterilize female white mice. However, with one exception (Experiment 4) larger doses were effective. The apparent anomaly when a dose of 6.8 millicurie hours was used (Experiment 4) is due to the fact that in this case the emanation tubes were placed directly over the heads of the animals (Table I). Thus the ovaries were at a greater distance from the radioactive source, and they also were better protected from the radiation by a greater thickness of tissue in the path of the rays. In Experiment 6, the dose, 21.9 millicurie hours, was sufficient to cause sterilization, even though

bondeau, L., and Récamier, D., *Compt. rend. Soc. biol.*, 1905, xii, pt. 1, 284.  
Brown, T., and Osgood, A. T., *Am. J. Surg.*, 1904-05, xviii, 179.

the mode of application was the same as in Experiment 4. The controls of either sex were all fertile. The first litter of young from both radiated and non-radiated female mice was obtained between the 60th and 78th day after birth. The radiated males reached sexual maturity at about the same time as the controls. The young born from the radiated mice grew normally and in turn produced normal

TABLE III.  
*Effect of Radium Radiations upon Longevity of White Mice.*

Experiment No.	Group.	No. of mice used.	Days after birth.	No. of mice alive.	Days after birth.*	No. of mice alive.
1	Control. 1.9 millicurie hours.	4	384	4	587	2
		3		3		1
2	Control. 2.4 millicurie hours.	4	327	0		
		4		0		
3	Control. 4.9 millicurie hours.	3	485	2	602	1
		4		3		1
4	Control. 6.8 millicurie hours.	2	186	2	Discontinued.	
		3		3		
5	Control. 11.5 millicurie hours.	5	502	5	618	3
		6		5		3
6	Control. 21.9 millicurie hours.	4	404	4	607	1
		2		2		2
7	Control. 26.8 millicurie hours.	4	500	2	616	2
		4		3		2

\* Final observation made November 20, 1921.

young, judging from their general appearance, weight, and reproductive power.

Davey<sup>7</sup> has reported some experiments with *tribolium confusum* in which by small doses of x-rays he was able to prolong the average life of the beetles. He used a very large number of specimens, and the statistical results are quite reliable. From our experiments, however, no definite conclusions can be reached as to the effect of radia-

tion on the longevity of white mice. The number of animals used was too small to apply statistical methods, and some of the mice are still living. It is interesting to note, however, that even those mice which showed severe local and constitutional effects from the radiation apparently did not have their lives shortened appreciably. Some were a little undersized but otherwise apparently normal on the 618th day. Table III shows the number of mice still living at two different periods in the course of the experiments.

#### DISCUSSION AND SUMMARY.

It has been estimated<sup>12</sup> that 92 per cent of the total radiation emitted by radium in equilibrium with its subsequent products is given off in the form of  $\alpha$ -rays. This, however, cannot be utilized when the source is enclosed in an ordinary container, because the  $\alpha$ -rays are absorbed completely by even a small thickness of glass. About 3.2 per cent of the total radiation is emitted in the form of  $\beta$ -rays, and 4.8 per cent as gamma radiation. The effects produced on the radiated mice of these experiments were due mainly to the  $\beta$ -rays, which are easily absorbed by tissue. The  $\gamma$ -rays, being only slightly absorbed by organic matter, probably contributed very little to the observed effects.

It is interesting to correlate the different effects produced by the same dose of radiation. The mice which received a dose of 1.9 millicurie hours showed no local effects on the skin or hair. Neither females nor males were sterilized, and the time at which they opened their eyes or reached sexual maturity was not affected, as far as we could tell. The only difference noted between the radiated animals and the controls was in the body weight. This dose accelerated the growth of the young mice, that is, while initially of the same weight, soon after irradiation they became distinctly bigger than the controls, but finally the animals of each group had substantially the same average weight. That this variation in body weight should be accidental is unlikely, since it was observed also in the animals treated by a slightly larger dose (2.4 millicurie hours). The number of animals

<sup>12</sup> Rutherford, E. *Radioactive substances, and their radiations*, Cambridge, 1913, 581.

(seven) which showed this effect is too small to prove conclusively the accelerating effect of small doses of radiation on the body growth of mice. But considering that similar results have been obtained by radiating plants<sup>6,7</sup> and beetles,<sup>7,8</sup> it is reasonable that the observed increase in weight might be attributed, at least in part, to the effects of radiation. Since this paper was first written Russ, Chambers, and Scott<sup>13</sup> have shown that small doses of x-rays accelerate the body growth of rats. In view of this additional evidence there can be little doubt that the increase in weight observed in our experiments was due to the radiation.

A dose of 2.4 millicurie hours applied over the backs of the animals produced no local skin effects, but it accelerated the growth of the mice as in the previous case. In addition it caused permanent sterilization of all the females. A similar result was obtained with 4.9 millicurie hours, except that the effect on the rate of growth was uncertain. A dose of 6.8 millicurie hours produced a definite but mild skin erythema and retarded the development of lanugo hair. But since in this instance the emanation was applied over the heads of the animals, the dose reaching the ovaries was not sufficient to cause sterilization, as already explained. No other definite effect was noted.

In connection with the sterilization of the females it should be noted that a dose of radiation which produced no visible skin changes was sufficient to cause permanent sterility. On account of the greater distance of the ovaries from the source of radiation as compared with that of the skin directly below the tube, and the depth of tissue which the rays had to traverse to reach the ovaries, the amount of radiation acting on the latter was much smaller than the amount falling on the skin. The radiation emitted by the emanation tube is reduced to about 50 per cent of its initial value after traversing 1 mm. of tissue. Still, while the skin was not visibly affected, the mice were sterilized. This shows that the ovaries are influenced very easily by radiation of this type. We can estimate the amount of radiation reaching the ovaries which is sufficient to cause sterility to be less than 25 per cent of the amount necessary to produce visible skin changes in the mice. It should be noted also that whenever sterility of the female mice was

<sup>13</sup> Russ, S., Chambers, H., and Scott, G. M., *Arch. Radiol. and Elect.*, 1921, xxvi, 128.

induced, it was permanent. Furthermore, those mice which were not rendered sterile by radiation were, as far as the experiments enable us to say, as prolific as the controls. Remembering that a dose of 1.9 millicurie hours had no apparent effect on the ovaries, while a slightly larger dose, 2.4 millicurie hours, caused permanent sterility, it might be concluded that it is not possible to produce temporary sterility by radiation. We know, however, that temporary sterility can be produced, at least when the animals are radiated at a later stage in their development. The mice in our experiments were radiated for the first time soon after birth, and it is not improbable that under these conditions temporary sterility cannot be obtained.

Large sublethal doses produced severe skin burns, retarded the body growth of the animals, but failed to sterilize the males. About one-third of the total skin area of the mice showed marked effects from the radiation. The animals were very sick for a time, and their growth was temporarily stunted. But nevertheless they recovered and finally became apparently normal except for the narrow hairless strip of skin which had been closest to the emanation tube. Only the females were rendered permanently sterile. The males did not show even temporary sterility when the doses of radiation were close to the lethal dose. While the testes of mammals are known to be very easily affected by radiation, still they are more resistant than the ovaries. In addition, in these experiments they were at a greater distance from the source of radiation than the ovaries, and they were better protected by the thicker layer of tissue in the path of the rays. The fact that no sublethal dose in these experiments sterilized the males shows that under the conditions of irradiation adopted the amount of radiation reaching the testes was not sufficient to affect them noticeably. If the source of radiation had been applied closer to the reproductive organs of the males, they would have been sterilized by millicurie hour doses much smaller than the lethal dose.

Some of the radiated animals were killed with ether, and macroscopic and microscopic examinations of the reproductive organs were made. The ovaries of the sterile females were generally atrophied and colored yellow. The normal histological structure was altered. The characteristic findings were the destruction of the Graafian follicles,

with absence of ovum cells. The testes and the epididymis of the radiated mice of the present experiment appeared macroscopically and histologically normal, with the presence of abundant spermatozoa. Owing to the method adopted for the irradiation of the mice, the testes were too far from the source of radiation, and too well protected by the intervening tissue to be definitely affected by the rays.

## BANANA GEL.

By GRACE MCGUIRE AND K. GEORGE FALK.

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(Received for publication, January 12, 1922.)

### INTRODUCTION.

The change of a soluble sucrase preparation from bananas into an insoluble preparation was described in a previous paper.<sup>1</sup> This change was found to occur on dialysis against running tap water. The insoluble form showed many of the characteristics of a gel. In this paper, a more complete study of the factors influencing the formation of this gel will be presented together with some of its properties but without considering its possible connection with enzyme activity.

### EXPERIMENTAL.

#### *Methods.*

The banana extract was prepared by passing the pulp of ripe bananas through a fine food-chopper, mashing it in a mortar with the requisite amount of water or sodium chloride solution, and filtering through paper in large funnels. Toluene was added before the filtration. The extract had a brown tinge. It had a pH value in the neighborhood of 5.0. Bananas in the same stage of ripeness were used as far as possible. Individual minor variations in the gelling properties of extracts were observed at times, just as the constituents of bananas may differ to small extents.

Collodion bags were used for dialysis. These were prepared in the usual way but were not standardized. However, the conditions for their preparation were kept uniform so that possible errors from this source need not be considered in the present connection. The volume of liquid in any one bag was always less than one-fourth the capacity of the bag, to allow for the increase in volume due to the dialysis.

<sup>1</sup> Falk, K. G., and McGuire, G., *J. Gen. Physiol.*, 1920-21, iii, 595.

Hydrogen ion concentrations were determined by use of indicators and suitable standard solutions.

The various salts which were used were either purified, or the impurities determined, since in the work to be described, minute amounts of certain substances were found to produce striking phenomena.

### *Results.*

#### *I. Dialysis of Banana Extract against Tap Water and against Distilled Water.*

The results obtained in a large number of experiments in the dialysis of banana extracts may be summarized briefly in the following paragraphs. Essentially the same results were obtained whether the extracts were prepared with water or with molar sodium chloride solution. As a rule, in the preparations 1 part by weight of liquid was used with 4 parts of banana pulp.

##### *1. Dialysis of extract against tap water.*

(a) Gel formation began in 24 to 48 hours. With small volumes more rapid changes including formation of gel occurred than with large volumes.

(b) Volume increased about 100 per cent. Most of the increase occurred before gel formation began. Changing bags during the dialysis had no effect.

(c) After two or three days dialysis the liquid still had a pH of 5.0 to 5.5.

(d) Gel formation began on the inner walls of the bags.

(e) Continued dialysis caused the dark colored gel to flake or separate out, leaving the liquid clear and colorless. As the flakes were formed, the pH of the supernatant liquid was 5.0 to 5.5 at first, but after 4 to 6 days became that of the tap water (pH 7.0).

##### *2. Dialysis of extract against distilled water.*

(a) No gel formation at any time.

(b) Continued increase in volume. In one experiment an increase from 25 cc. to 178 cc. in 4 days was observed.

(c) After dialysis pH of liquid unchanged (about 5.0).

(d) Liquid became cloudy and light colored (perhaps because of dilution).



3. *Dialysis of gel from 1 (e) against distilled water.*

(a) Gel disappeared on continued dialysis (up to 6 days) against renewed distilled water.

(b) Increase in volume as in 2 (b) in every case.

(c) Liquid became cloudy and lighter colored as in 2 (d) if the gel disappeared.

4. *Dialysis of liquid from 2 (d) against tap water.*

(a) Cloudiness disappeared.

(b) No further increase in volume.

(c) Some flakes of gel separated.

Toluene was present in all these experiments. However, long continued shaking of the extracts with toluene produced no gel. Bubbling air through the extracts also did not result in gel formation.

Most of the experiments were carried out by dialyzing against running tap water or distilled water. Some experiments dialyzing against tap water or distilled water in large beakers gave the same results.

The difference in the behavior of the banana extract relative to the gel formation upon dialysis against tap water and distilled water, was evidently due to differences in the compositions of the two. The pH of the tap water was found repeatedly to be 7.0 to 7.2; that of the distilled water about 5.0. An average analysis of the inorganic constituents, as parts per million of the tap water was as follows:<sup>2</sup>

CaCO <sub>3</sub> .....	24	NaCl.....	5
MgCO <sub>3</sub> .....	5	KNO <sub>3</sub> .....	1
MgSO <sub>4</sub> .....	10	SiO <sub>2</sub> .....	9

The distilled water was practically salt-free.

II. *Dialysis of Banana Extract against Solutions of Definite Hydrogen Ion Concentrations and Salt Contents.*

Banana extract was dialyzed for 48 hours at 10–15°C. in collodion bags against the following solutions contained in large beakers with the indicated results. Sodium hydroxide or hydrochloric acid were

<sup>2</sup> The analysis was obtained through the courtesy of Mr. Merritt H. Smith, Chief Engineer of the Department of Water Supply, Gas and Electricity, New York City.

used when necessary to produce the required pH values. That sodium chloride did not play a positive part in the gel formations was evident from the fact that the banana extracts in many experiments were prepared with sodium chloride solution.

When gel formation occurred, the volume increases were 100 per cent or less, when no gel formation occurred the volume increases ranged from 100 to 400 per cent in 48 hours. In a number of experiments, the pH of the outside liquid changed from 7.0 to 6.0 or even less. Whenever this occurred, no gel was formed inside the bags.

These results showed that for the formation of a gel from banana extract by dialysis against tap water, the presence of a calcium salt

Solutions.	pH of outside liquid.	Results.
CaCO <sub>3</sub> 0.00020 to 0.00024 M <sup>3</sup> .....	7.0	Gel.
CaCO <sub>3</sub> 0.00020 to 0.00024 M; phosphate buffer <sup>4</sup> , 35 cc. : 2 liters.....	7.2	"
CaCO <sub>3</sub> Saturated, excess solid.....	7.0	"
CaCO <sub>3</sub> 0.00024 M.....	5.0	No gel.
Phosphate buffer, <sup>4</sup> 35 cc. : 2 liters.....	7.2	" "
MgSO <sub>4</sub> 0.00024 M.....	7.0	" "
MgSO <sub>4</sub> 0.00024 M.....	5.0	" "
Ca(OH) <sub>2</sub> Saturated, excess solid CaO.....	More alkaline than 10.0	Gel (white).

and an alkalinity corresponding to pH 7.0 or more were the important factors.

In order to determine whether the collodion bag as such played a part in the gel formation or whether possibly interfering substances were removed by dialysis, the following experiments, in which a number of different salts were added directly to banana extract at different hydrogen ion concentrations, were carried out.

### *III. Actions of Salts on Banana Extract at Definite Hydrogen Ion Concentrations.*

No satisfactory quantitative methods for the comparison of gels, the readiness of their formation, and their chemical compositions,

<sup>3</sup> 20 to 24 parts per million.

<sup>4</sup> Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, 76.

are at hand. In the following experiments, in comparing different gels, their consistency is taken to be the greater or smaller resistance to deformation, the ability to retain the shape of the vessel in which they were prepared, even when separated from it, etc. In any one series a satisfactory comparison can be made, but in different series carried out at different times, anything more than a rough qualitative study is difficult.

Since calcium salts were found to be involved in the gel formation in the dialysis experiments, a number of series of experiments were carried out in which solutions of calcium salts were added directly to banana extracts under different conditions without dialyzing.

The acidity of the mixture was the first determining factor. A banana-water extract (4 parts of banana pulp with 1 part of water and filtered in the usual way) gave no indication of gel formation at the pH of the juice (about 5.0) with 0.29 mg. of calcium, added as calcium oxide, or 0.22 mg. of calcium, added as calcium chloride, per cc., in 48 hours at 5°–10°C. The same extract at pH 7.5 with 0.04 mg. of calcium as calcium oxide added per cc. formed a firm gel in 5 minutes which retained the shape of the container. These results were confirmed repeatedly. It appeared to be impossible to obtain gel formation with calcium salts with solutions more acid than about pH 6.0. With solutions at pH 7.0 or more alkaline, gels were obtained readily. The greater the concentration of calcium the more rapid the formation of a firm gel, and *vice versa*. For example, with a calcium concentration of 0.03 mg. per cc. and the juice mentioned above, gel formation had only begun after 20 minutes, while a firm gel was present in 7 hours. With 0.02 mg. of calcium per cc. at 5° in 18 hours, definite gel formation had occurred, while with 0.01 mg., the gel formation was doubtful. Low temperatures favored the formation of the gel. While extracts obtained at different times differed slightly, the general conclusion that with calcium salts no gel formation at pH 6.0 and more acid, and gel formation at pH 7.5 and more alkaline, was found to hold in every case.

It is of interest to note that if the banana extract is boiled for a few minutes, some solid shreds forming, a gel could not be obtained either by dialysis against tap water or at pH 7.5 by the addition of calcium salts.

These results are of importance in connection with the preparation of banana extract and subsequent treatment of the latter. As described previously, the banana extract was prepared with sodium chloride solution for a number of experiments. It was found necessary to purify the sodium chloride, since a number of samples of the latter were found to contain small quantities of calcium salts. If the calcium was not removed, on bringing the extract to pH 7.0 or more alkaline condition for various purposes, gel formation would take place very rapidly. Similarly, if sodium hydroxide which contained calcium as impurity, was used for neutralization, gel formation might occur. The results described above were therefore obtained by neutralization with ammonium hydroxide. An added complication was found in the fact that the banana itself contains very small amounts of calcium salts<sup>5</sup> which may appear in the extracts. Long standing (48 hours or more) in solutions of water extract brought to pH 7.0 with ammonium hydroxide resulted in the formation of some gel presumably from this cause.

Sodium chloride extracts showed smaller gelling actions with calcium salts than did water extracts. Thus, with two banana extracts, one prepared with water, the other with sodium chloride solution, treated with 0.02 mg. of calcium in the form of calcium chloride per cc. under the same conditions, formed a firm gel in the first case in 10 minutes, and very little gel in the second.

A comparative study of the gelling actions of certain salts was made. Two series were run, one at pH 5.0, and the other at pH 7.5. To 30 cc. portions of banana-water extract were added 0.5 cc., 1.0 cc., and 1.5 cc. of 0.044 M solutions of calcium chloride, strontium chloride, barium chloride, magnesium chloride, and lithium chloride. None of the solutions at pH 5.0 gelled even after 48 hours. Of those at pH 7.5, the calcium chloride showed the most marked gelling action, the strontium chloride showed somewhat less, and the barium chloride still less. The magnesium chloride and lithium chloride gave no indication of gel formation until after 7 hours, after 48 hours there were soft gels formed but even less than that formed in the control, water extract plus ammonium hydroxide to pH 7.5. The relations

<sup>5</sup> Colby, G. E., *California Agric. Exp. Sta. Report*, 1892-94, 275.

in the gel formations with time and concentration of salt with the first three salts were similar to those already given.

#### *IV. The Action of Pancreatine on Banana Extract.*

As a result of some experiments carried out for a different purpose, the formation of a gel by the action of pancreatine on banana extract was observed. For example, one 40 cc. portion of banana sodium chloride extract was treated at pH 5.0 with 0.2 gm. of a commercial pancreatine preparation, and another with 0.1 gm. In 24 hours at 38°, the former formed considerable gel, the latter formed less gel. At 5°, the same mixtures produced no gels. If either the pancreatine or the extract was boiled before mixing the solutions, no gel was obtained. The gel which was obtained by the action of the pancreatine appeared to be less firm than that obtained by the action of calcium salts.

A number of the gels prepared by dialysis were filtered on paper, washed with alcohol and ether and dried and analyzed. The analyses, however, mean nothing. The gels as obtained carried down mechanically or otherwise much of the dissolved material present. Attempts to wash the gels with water were not successful, swelling occurring in almost every case. Until methods of purifying the gels, or at least of removing extraneous material are developed, nothing definite can be stated relative to their chemical composition. It may be mentioned, however, that the nitrogen content of the dried gel was in the neighborhood of 4.5 per cent, pointing to the presence of about 25 per cent protein material.

#### DISCUSSION.

The question of the relation of this work to other work of similar nature can be disposed of briefly. The source of the material and some of the reactions may indicate that the gels are of the nature of pectins.<sup>6</sup> However, against this view are the facts that boiling destroys the gel-forming property and that only very small quantities of calcium and strontium salts are required to yield the gels under suitable conditions.

<sup>6</sup> Cf. Haynes, D., *Biochem. J.*, 1914, viii, 553.

The formation of the gels by the direct addition of certain salts under suitable conditions as well as by dialysis against solutions of such salts is significant. It raises the question whether it would not be possible in many cases to bring about reactions such as precipitation, solution, etc., by direct chemical actions involving the addition of reagents under definite conditions, in place of dialysis against solutions of more or less accidental salt contents. The fact that such solutions as the tap water which was used in the present investigation may contain dissolved substances in minute concentrations only serves to obscure the possible actions. These small concentrations of dissolved substances which may react, but which are constantly renewed as the dialyzing liquid changes, are capable of producing effects which larger initial concentrations would produce in shorter times. Thus, the banana gel-forming substance inside the bags was able to react with the calcium salts from the outside liquid and over the extended period of time which was used here and is also used in most dialysis studies, the reaction occurred, first along the inner walls of the bags and finally throughout the liquid. The different hydrogen ion concentrations inside and outside the bags also served to confuse the exact conditions at which the reactions could take place.

These relations make it advisable in every case in which dialysis is used to study the composition of the dialyzing liquid, as small amounts of dissolved substances and also apparently unimportant differences in hydrogen ion concentrations may result in the occurrence of definite chemical changes. Also, the material of which the dialyzing bags are made may exert profound effects upon the changes.<sup>7</sup> The plea may be made, therefore, to replace dialysis wherever possible by direct additions of chemical reagents at the same time controlling the hydrogen ion concentrations of the mixtures, and in this way substituting more or less accidental and to some extent unknown methods of treatment by known and definite additions of chemical reagents.

The results obtained therefore point to chemical reactions and combinations involving specific elements under definite and limited conditions. Although it was not found possible to obtain these compounds in pure condition as chemical individuals, the results can best be interpreted on the basis of the formation of such chemical compounds.

<sup>7</sup> Brown, W., *Biochem. J.*, 1915, ix, 591; 1917, xi, 40. Eggerth, A. H., *J. Biol. Chem.*, 1921, xlviii, 203.

## SUMMARY.

The conditions for the formation of gels from banana extracts were studied.

Gels were obtained with extracts more alkaline than pH 7.0 with very small quantities of calcium, strontium, and barium salts, the gel formation with these salts decreasing in the indicated order.

In solutions more acid than pH 6.0, no gels were obtained with these salts.

Magnesium, lithium, and sodium salts did not cause gel formation either in acid or alkaline solutions.

Pancreatine gave a gel on incubation with banana extract at pH 5.0.

The gel-forming property of banana extracts was destroyed on boiling.

We wish to thank the Fruit Dispatch Company for supplying the greater part of the bananas used in this investigation.





## QUANTITATIVE LAWS IN REGENERATION.

### III. THE QUANTITATIVE BASIS OF POLARITY IN REGENERATION.

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(Received for publication, January 18, 1922.)

#### I. INTRODUCTION.

It has been shown in preceding papers that the dry weight of shoots and roots produced under equal conditions of illumination, moisture, and temperature in sister leaves of *Bryophyllum calycinum* varies approximately in direct proportion with the dry weight of the leaves; and that the same is true for the mass of shoots produced in small pieces of stem connected with a leaf.<sup>1</sup> It had been known that when a piece of stem is left in connection with a leaf, the mass of shoots produced by the leaf is less than when the leaf is completely isolated, and the writer had been able to show that in this case the stem connected with the leaf gains approximately as much in dry weight as the dry weight of the shoots and roots in the leaf would have been if the leaf had been completely isolated from the stem.<sup>2</sup> The inhibitory influence of the stem on the shoot and root formation in the leaf was in this case due to the fact that when the leaf is connected with a stem, that part of the material which could have been utilized for the formation of new shoots and roots in the leaf now goes into the stem. It is intended to show in this paper that the same simple quantitative relations suffice to account for the polar character of regeneration in a defoliated stem of *Bryophyllum*.<sup>3</sup>

The reader will remember that each node of the stem of this plant has two dormant buds capable of growing into shoots. When a piece of defoliated stem is cut from a plant and suspended in moist air, only

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 81; 1919-20, ii, 297, 651. *Science*, 1917, xlv, 436. *Bot. Gaz.*, 1918, xlv, 150. *Ann. Inst. Pasteur*, 1918, xxxii, 1.

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 297, 651.

<sup>3</sup> Loeb, J., *Science*, 1921, liv, 521.

the two buds of the most apical node will grow into shoots, while the buds in all the nodes below will remain dormant. Permanent roots will grow only at the base of each piece, though transitorily air roots

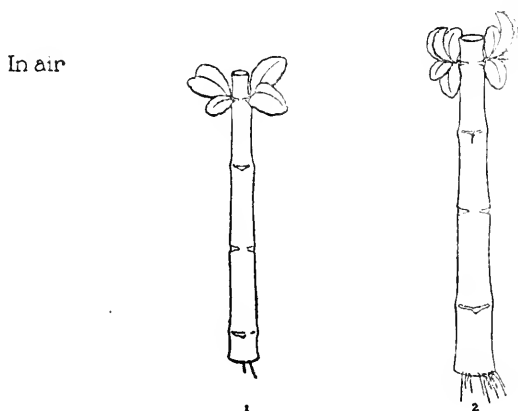


FIG. 1. Pieces of stem from the same plant, (1) apical, (2) basal. Suspended in moist air, shoots formed only in the apical node, roots at the base. Mass of shoots and roots is larger in the basal piece (2) which has the larger mass. Duration of experiment October 4 to November 7, 1921.

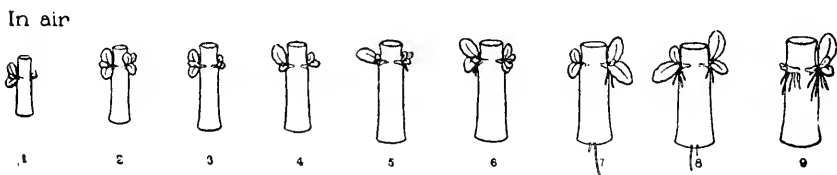


FIG. 2. Stem cut into small pieces with 1 node each. Suspended in same aquarium and simultaneously with large stems in Fig. 1. (1) was the most apical, (9) the most basal piece, the serial number denoting the original position of the pieces in the plant. Each piece of stem forms 2 shoots in its node, but the relative mass of the shoots varies with the relative mass of the stem, not with the serial number of the node.

may begin to form in any node, but these will dry out as soon as the basal roots are growing.<sup>4</sup> Fig. 1 illustrates this polar character of regeneration in defoliated pieces of stem suspended in moist air. When, however, a long defoliated stem is cut into as many

<sup>4</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 687.

pieces as there are nodes, then all the dormant shoot buds of the stem will grow out into shoots (Fig. 2). The stems in Figs. 1 and 2 were cut out at the same time and suspended in moist air in the same vessel.

The results remain about the same when the basal ends of the pieces are dipped into water, the only difference being that often not only the

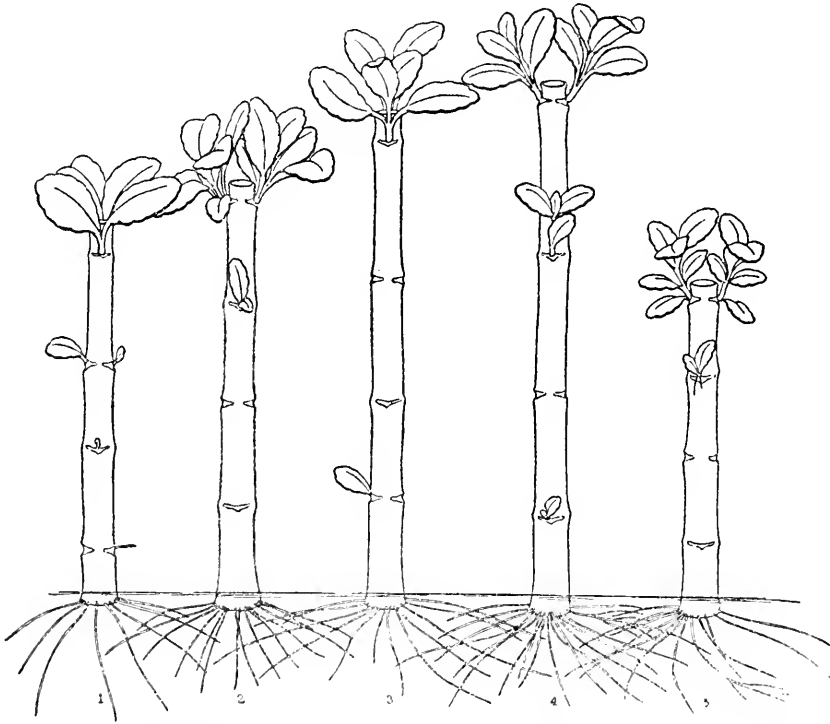


FIG. 3. Same experiments as Fig. 1, only that the long pieces of stem were put with their bases into water. Duration of experiment from September 27 to October 22, 1921. All stems were cut from one plant.

two buds in the most apical node of a long piece of stem grow out but also one or two buds of the node below (Fig. 3). The amount of growth of shoots and roots is also greater in the stems put with the base in water (Fig. 3) than when the stems are suspended in moist air (Fig. 1). When pieces of stem with only one node each are put into water, each piece forms shoots at its node (Fig. 4). The

question is, Why do only the most apical buds of a long defoliated stem grow out? Bonnet had suggested that the ascending sap of a plant was shoot-producing and the descending sap was root-producing. Sachs pointed out that when a piece of stem was cut out from a plant the ascending sap was blocked at the apex and that hence the shoot-producing substances must collect at that end of the stem giving rise to shoots at the apical node; while the descending sap is blocked at the base, giving rise to root formation at that end.

The problem then exists to prove or disprove the old suggestion of Bonnet and Sachs. The formation of shoots or roots is a synthetic process or a series of catenated synthetic processes, in which soluble

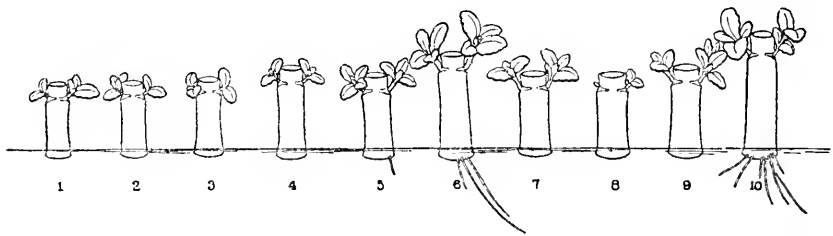


FIG. 4. Stem of one plant cut into ten small pieces, the serial number indicating their position in the plant, (1) being the most apical, (10), the most basal piece. Base in water. Experiment simultaneous with Experiment III. Each piece has formed 2 shoots the relative size of which does not follow the serial number of the stem, but the relative size. The size of each shoot of the pieces is much smaller than the size of the shoots formed simultaneously by the larger stems in Fig. 3. The latter stems all have roots, while only the two largest pieces of stems (6) and (10) in Fig. 4 have formed roots.

materials, such as sugars, amino-acids, and other substances, are synthetized into the larger molecules of proteins, compounds of the cellulose type, and others. If the theory of Bonnet and Sachs is correct, it must be possible to show that the two shoots formed at the apex of a long defoliated piece of stem have, within the limits of the accuracy of the experiments, approximately the same dry weight as the dry weight of all the shoots would have amounted to if the stem had been cut into as many small pieces as it contained nodes.

By comparing the amount of shoots formed in the one-node pieces in Fig. 2 or 4 with those of the four-node pieces in Fig. 1 or 3, the reader will notice that the shoots are greater in the larger pieces of

stem, and the same fact is obvious from all the other figures in this paper. It is almost obvious from a glance at the figures that the mass of shoots formed increases with the mass of the stem. If the mass of shoots produced at the apex of large pieces of stem is approximately equal to the mass of shoots which the same stems would have produced had they been cut into as many pieces as the stems contained nodes (*i.e.*, into one-node pieces), it will be necessary to show that within the limits of the experimental errors, the mass of dry weight of shoots produced per gram of dry weight of stem is about the same regardless of whether the stems are long or whether they are subdivided into one-node pieces.

This was tested in various ways. The defoliated stem of a very large plant was cut into 5 pieces, each possessing 4 nodes (Fig. 3), and the defoliated stem of a second plant was cut into 10 small pieces of 1 node each (Fig. 4). The pieces dipped with the base into water and the large and small pieces were suspended in the same aquarium. The experiment lasted from September 27 to October 22, 1922. The shoots were then cut off and both shoots and stems were dried for 24 hours in an oven at about 100°C. The result was as follows: The dry weight of the 5 large stems (Fig. 3) was 13.670 gm., and the dry weight of their 16 shoots was 0.495 gm. The shoot production was therefore 36 mg. per gram of stem (all measured in dry weight). The dry weight of the 10 short pieces of stem with 1 node each (Fig. 4) was 2.880 gm., and the dry weight of 19 shoots was 0.115 gm., or 1 gm. of dry weight of stem produced 40 mg. of dry weight of leaves. These two figures, 40 mg. and 36 mg., agree sufficiently closely to show that under equal conditions the production of shoots of defoliated pieces of stem occurs in proportion with the mass of the piece of (defoliated) stem; or, in other words, the mass of shoots produced at the apex of the large defoliated stems of Fig. 3 is approximately equal to the mass of shoots the same stems would have produced if all the dormant buds of each stem had been able to grow out.

The experiment in Figs. 1 and 2 gave a similar result. The experiment lasted from October 4 to November 7. 5 large stems with 4 nodes each (Fig. 1) having a dry weight of 5.486 gm. produced 10 shoots with a dry weight of 0.114 gm.; *i.e.*, 20.8 mg. of shoot per gram of stem.

4 short pieces of stem with 2 nodes each, having a dry weight of 3.214 gm., produced 8 shoots with a dry weight of 0.0668 gm.; *i.e.*, 20.7 mg. of shoot per gram of stem.

A third stem was cut into 9 pieces with 1 node each (Fig. 2) possessing a dry weight of 3.270 gm., giving rise to 17 shoots with a dry weight of 0.050 gm.; *i.e.*, 15.3 mg. of shoot per gram of stem.

The first two figures are identical, the last figure is a little low. In these experiments the end of the piece may suffer (by drying out or falling a prey to fungi) and this creates an error which is especially noticeable when a stem is cut into many small pieces. But in spite of these sources of error the results are remarkably clear and consistent.

It seemed of interest to compare the behavior of defoliated stems split longitudinally. In this case the two halves should give approximately equal results.

## *II. Experiments with Split Stems.*

Experiments were made with stems split longitudinally as indicated in Fig. 5. Only pieces from the middle of the stem of a large plant were used, for reasons to be given later. Stems with 4 nodes each, were split longitudinally and one half was cut transversely into 2 pieces with 2 nodes each, *a'*, *b'*, and *c'*, *d'*, respectively (see Fig. 5). The other half with the 4 nodes *a*, *b*, *c*, and *d* was not cut transversely. All 3 pieces (Fig. 5) were put with their bases into water. It was to be expected that the sum of the dry weight of the shoots produced by the 2 small pieces with 2 nodes each should equal the dry weight of the shoots produced by the larger pieces with 4 nodes each. Fig. 5 shows at a glance that this is approximately the case and the dry weight determinations confirm this.

The first experiment was carried out on 7 stems, a second experiment on 16 stems. Table I gives the result.

It is therefore obvious that the dry weight of the sum of the shoots produced by the small pieces *a'*, *b'*, and *c'*, *d'*, approximately equals the dry weight of the shoots produced by the big pieces, *a*, *b*, *c*, and *d* (Fig. 5), or, in other words, the mass of shoot produced at the apex of the large pieces is approximately equal to the dry weight of the shoots the same stems would have produced if the buds of every second node had been able to grow.

TABLE I.

Experi- ment No.	Duration of experiment.	Number of pieces.	Dry weight of shoots produced.	Dry weight of stems.	Shoots produced per gram of stem.
	1921		gm.	gm.	mg.
I	Nov. 3– Dec. 6.	7 four-node pieces, <i>a, b, c, d.</i>	0.1545	4.290	36.0
		14 small pieces, <i>a', b', c', d'.</i>	0.147	3.822	38.7
	1921-22				
II	Dec. 8– Jan. 10.	16 four-node pieces, <i>a, b, c, d.</i>	0.750	16.646	45.0
		32 two-node pieces, <i>a', b', c', d'.</i>	0.577	14.527	39.5

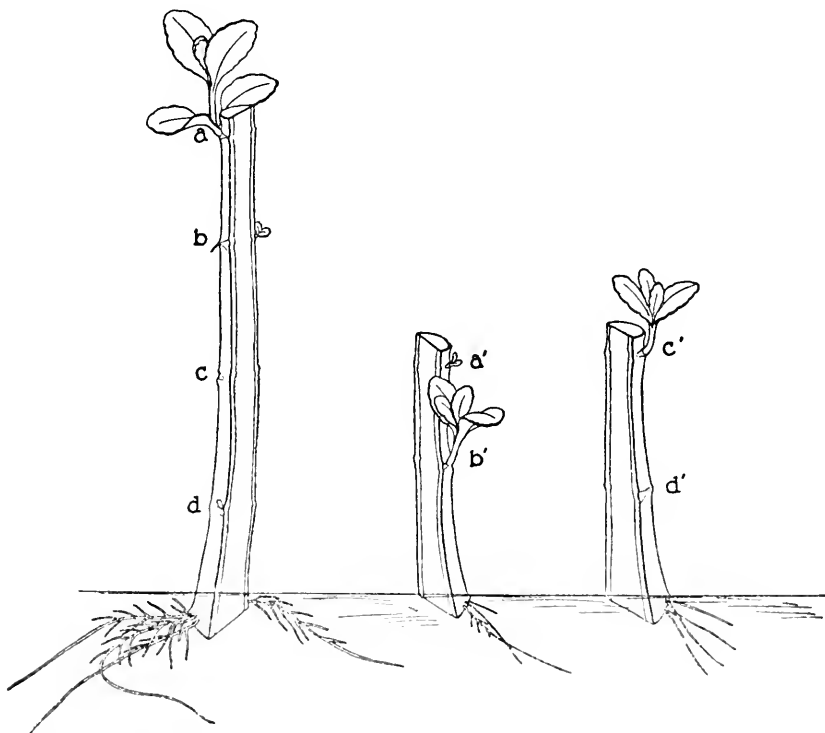


FIG. 5. Piece of stem with 4 nodes, *a, b, c, d*, split longitudinally. One half cut transversely into two pieces, *a', b'*, and *c', d'*. The half *a, b, c, d*, produces 1 shoot which about equals in mass the 2 shoots produced by *a', b'*, and *c', d'*. Duration of experiment December 9, 1921, to January 4, 1922.

*III. Small and Large Pieces of the Same Stem.*

A third series of experiments was as follows: Long pieces of stem, containing about 10 nodes, were cut out from the same plant more

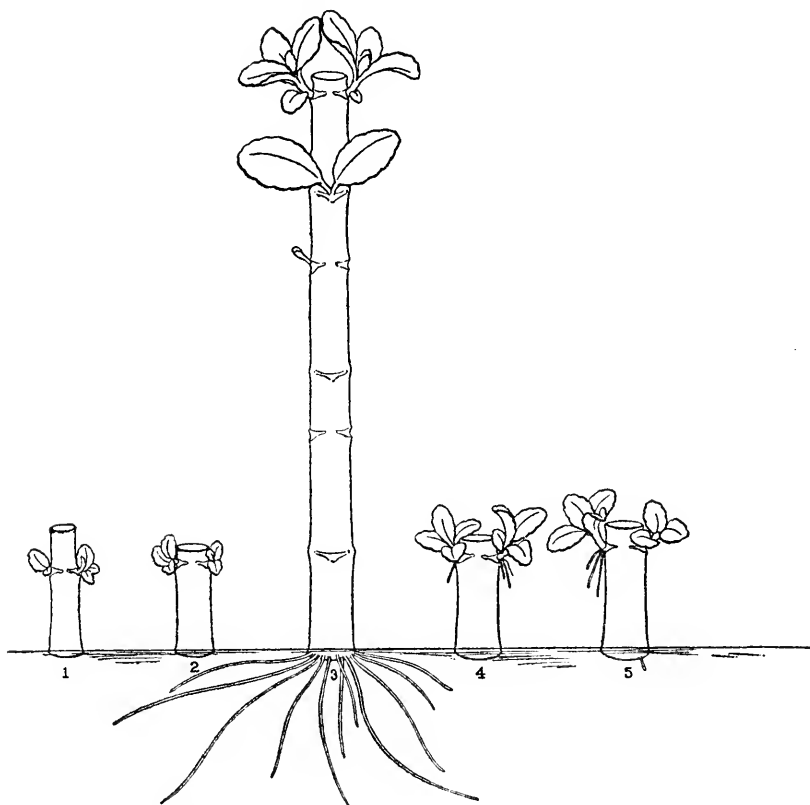


FIG. 6. Five pieces cut from the stem of the same plant, (1) and (2) apical pieces with 1 node each, (3) middle piece with 6 nodes, and (4) and (5) the basal pieces with 1 node each. The large middle piece produces larger shoots than either the more apical or more basal small pieces. The large middle piece has ample roots while only the longer basal piece commences to form a root. Duration of experiment October 25 to November 21, 1921.

than 1 year old (Fig. 6). The middle piece of about 6 nodes (piece 3 in Fig. 6) served for the experiment, two small pieces, 1 and 2, containing 1 node each and situated apically, and 2 small pieces, 4 and 5, also containing 1 node each, situated basally from the large middle



piece in the same stem, serving as controls. In other experiments of the same character, pieces containing about 14 nodes were cut out from the stem of the same plant; 2 small pieces at the apex, each containing 2 nodes (1 and 2, Fig. 7), and 2 small pieces at the base each

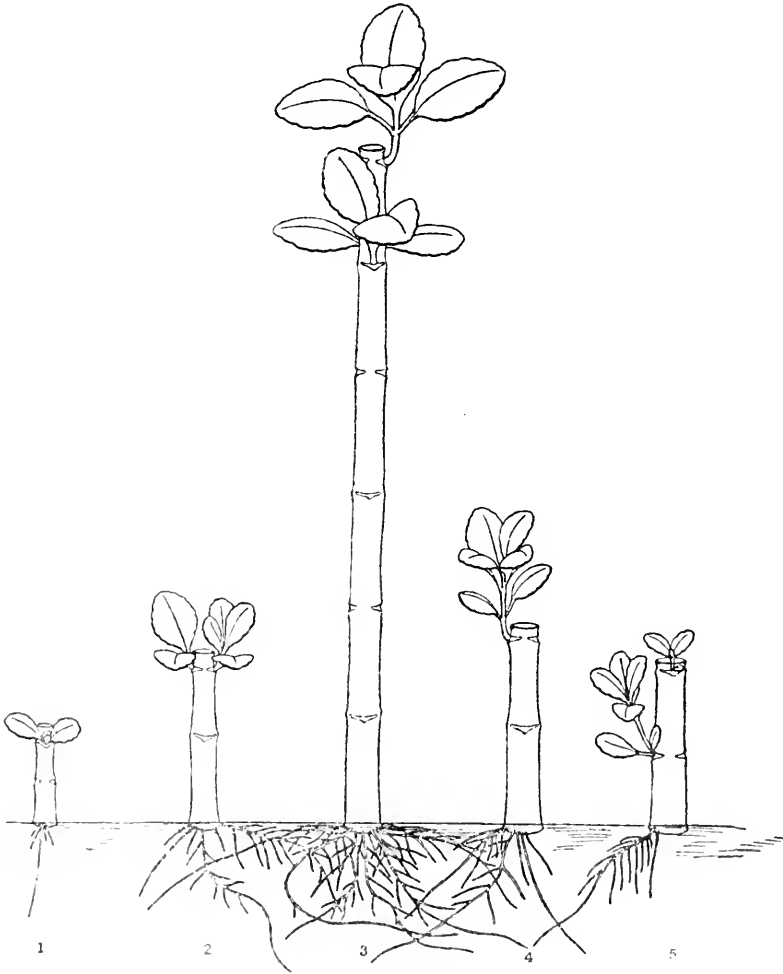


FIG. 7. Similar experiment as Fig. 6, except that the small pieces have 2 nodes each. Shoots and roots are formed in proportion to mass of stem. Duration of experiment November 16 to December 19, 1921.

containing also 2 nodes (4 and 5, Fig. 7), were used as controls, while the middle piece (3, Fig. 7) served for the main experiment. All the pieces dipped with their bases into water.

It is obvious from Fig. 6 that the large pieces of stem (3) produced larger masses of shoots than the small pieces 1 and 2 or 4 and 5 during the same time and under equal conditions. It may also be pointed out that these large middle pieces (3) formed their basal roots earlier than the small pieces (Fig. 6), and that the mass of their roots remained greater than the mass of roots in the small pieces (Fig. 7).

It turned out that the shoot production in the most apical pieces of stems 1 and 2 was usually irregular, as a rule too small, so that these pieces were not well usable as controls. The basal pieces, 4 and 5, however, behaved normally. It seems that this abnormal behavior of the small apical pieces is found as long as the leaves connected with this piece are still small and growing. It is therefore well to use in these experiments that part of the stem which is naturally defoliated or the leaves of which are about to fall. It may also be well not to use pieces of stem too near the roots. After 3 to 5 weeks the dry weight of the shoots and of the stem used in these experiments were determined. Since some of the small pieces of stem fall often a victim to fungi only one of the 2 small pieces, apical or basal, was used as a control.

*Experiment I. October 25, 1921, to November 25, 1921.*

	gm.	Dry weight of shoots per gram of stem. mg.
<i>6 long pieces with 6 nodes each.</i>		
Dry weight of stems.....	9.260	
“ “ “ 13 shoots.....	0.260	28.0
“ “ “ roots.....	0.057	
<i>Control a. 7 short basal pieces with 1 node each.</i>		
Dry weight of stems.....	2.895	
“ “ “ 13 shoots.....	0.088	30.4
“ “ “ roots.....	0.003	
<i>Control b. 12 short apical pieces with 1 node each.</i>		
Dry weight of stems.....	1.428	
“ “ “ 18 shoots.....	0.0236	16.5

It is obvious that the apical control pieces gave too small a production of shoots (16.5 mg. per gram of stem), while the basal con-

trol pieces produced approximately the same amount of shoots per gram of stem, namely 30.4 mg. as compared with 28.0 for the large pieces.

*Experiment II. November 2, 1921, to December 6, 1921.*

	gm.	Dry weight of shoots per gram of stem. mg.
<i>5 long pieces of stem with 6 nodes each.</i>		
Dry weight of stems.....	6.486	
“ “ “ 10 shoots.....	0.272	42.0
“ “ “ roots.....	0.0458	
<i>Control a. 4 short basal pieces with 1 node each.</i>		
Dry weight of stems.....	1.058	
“ “ “ 8 shoots.....	0.041	39.0
“ “ “ roots.....	0.0034	
<i>Control b. 5 short apical pieces with 1 node each.</i>		
Dry weight of stems.....	0.544	
“ “ “ 10 shoots.....	0.018	33.0

Again the short basal control pieces produce about as much shoot material per gram (39 mg.), as the large pieces (42 mg.), while the apical controls produce less, namely, 33 mg. We will omit the apical controls in the further tabulation of experiments on account of the irregularity of the results.

*Experiment III. November 16, 1921, to December 20, 1921.*

	gm.	Dry weight of shoots per gram of stem. mg.
<i>9 long pieces of stem with 6 nodes each.</i>		
Dry weight of stems.....	18.658	
“ “ “ 26 shoots.....	0.944	50.3
“ “ “ roots.....	0.1428	
<i>Control. 18 small basal pieces of 2 nodes each.</i>		
Dry weight of stems.....	18.147	
“ “ “ 36 shoots.....	0.800	44.0
“ “ “ roots.....	0.136	

*Experiment IV. October 22, 1921, to November 15, 1921.*

<i>4 long pieces of stem with 4 nodes each.</i>		
Dry weight of stems.....	4.214	21.0
“ “ “ 8 shoots.....	0.089	
<i>Control. 4 short basal pieces of 2 nodes each.</i>		
Dry weight of stems.....	2.492	19.0
“ “ “ 8 shoots.....	0.0475	

*Experiment V. October 11, 1921, to November 1, 1921.*

	gm.	Dry weight of shoots per gram of stem. mg.
<i>4 long apical stems with 6 nodes each.</i>		
Dry weight of stems.....	3.921	
“ “ “ 8 shoots.....	0.113	29.0
“ “ “ roots.....	0.0134	
<i>Control. 4 basal pieces of 2 nodes each.</i>		
Dry weight of stems.....	3.744	24.0
“ “ “ 10 shoots.....	0.090	

*Experiment VI. December 11, 1921, to January 17, 1922.*

<i>7 long apical stems with 6 nodes each.</i>		
Dry weight of stems.....	6.634	
“ “ “ 12 shoots.....	0.340	51.0
“ “ “ roots.....	0.0512	
<i>Control. 7 short basal pieces of 2 nodes each.</i>		
Dry weight of stems.....	3.560	
“ “ “ 12 shoots.....	0.1770	49.6
“ “ “ roots.....	0.0128	

If we consider only those figures in the experiments where the small control pieces of stem were situated basally from the long stem (the pieces 4 and 5 in Figs. 6 and 7), we notice that the differences of shoots produced per gram of dry weight of the controls differ never more than 25 per cent from those produced by the large pieces of stem and that in some cases the difference is only about 6 per cent. Considering the limitations in the experimental conditions—especially the fact that part of the stem may not function normally, especially the ends near the cut, or the fact that individual buds may have been injured by parasites, etc.—the agreement of the figures seems remarkable.

These results leave no doubt that within the limits of accuracy of these experiments the dry weight of the shoots produced at the apex of a long piece of defoliated stem is about equal the mass of shoots the same stem would have produced had the buds in all of its nodes been able to develop.

#### *IV. Regeneration of Roots.*

Two kinds of roots are formed in an isolated piece of stem, suspended in moist air and dipping with the base in water, first, air roots in the nodes, and later roots at the basal end of the stem regardless of the node (Figs. 1, 3, and 7). The air roots in the nodes grow out sooner than the basal roots but as soon as the basal roots grow out the air roots dry out and die. This has been discussed in a previous paper. We are interested here only in the basal roots since they alone are connected with the problem of polarity. The regeneration of the basal roots differs from the formation of apical shoots in this, that the apical shoots begin to grow out almost immediately after the defoliated piece of stem is isolated, while there is a long latent period before the basal roots make their appearance. For this reason quantitative measurements correlating the mass of the basal root formation with the mass of stem require probably a longer time than that in our experiments. A glance at the drawings will, however, convince the reader that the root formation commences sooner in the stems with larger mass than in the stems with smaller mass, regardless of the original position of the piece of stem in the plant.

Thus in Fig. 6 the large middle piece (3) forms roots before either of the 2 more basal pieces form roots, and Fig. 7 shows that the relative mass of roots produced seems also to run parallel with the relative mass of the piece. The same phenomenon is shown if we compare Fig. 1 with Fig. 2, or Fig. 3 with Fig. 4. It is also obvious in Fig. 5, so that we can say that the mass of roots produced by pieces of defoliated stem of *Bryophyllum calycinum* increases under equal conditions with the mass of the stem.

#### *V. Influence of Light on Regeneration in a Defoliated Stem.*

8 long defoliated stems were suspended into an aquarium kept dark by a double cover of black cardboard, and 8 equally long defoliated stems were put at the same time into an aquarium exposed in the usual way to daylight. The base of the stems dipped into water. All conditions were equal except the illumination. After 23 days all the stems exposed to light had formed large basal roots and large shoots at the apex (Fig. 8). At the same time none of the stems

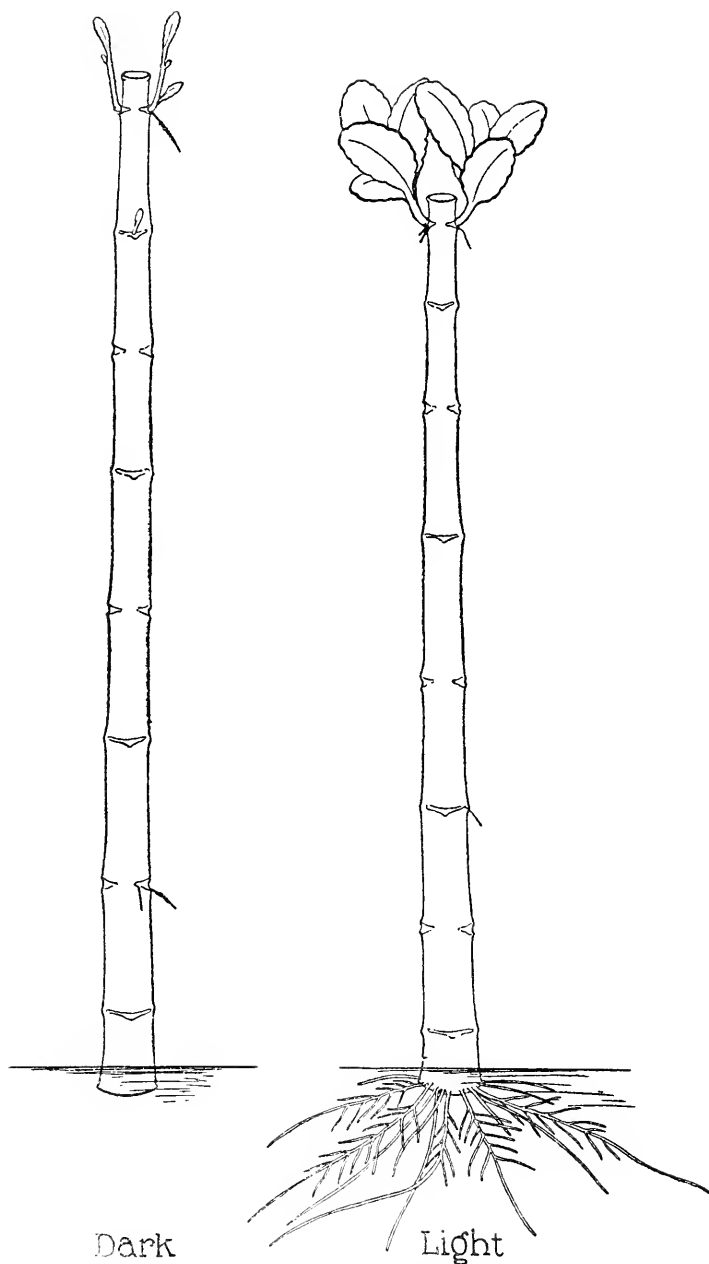


FIG. 8. Influence of light on root and shoot formation of stem. In the dark no roots are formed; in light ample roots are formed. The mass of shoots formed in dark is small compared with mass of shoots formed in light.

in the dark had formed a single basal root though some had formed tiny air roots (Fig. 8). The shoots formed in the dark had a small mass and the typical etiolated shape. The most striking phenomenon was the lack of root formation at the base of the stem in the dark. The writer had already shown that the favorable influence of the leaf on root formation in the stem also disappears when the leaf is deprived of light.<sup>4</sup>

#### SUMMARY AND CONCLUSION.

It is well known that a long defoliated piece of stem of *Bryophyllum calycinum* forms shoots only at the apical or the two apical nodes, while when such a stem is cut into as many pieces as there are nodes each node produces shoots. It is shown in this paper that the dry weight of shoots produced in the apical nodes of a long piece of stem is approximately equal to the dry weight of shoots the same stem would have produced if it had been cut into as many pieces as it possesses nodes. Hence all the material which can be used for the growth of shoots goes into the most apical part of the stem and this accounts for the polar character of regeneration in this case.<sup>5</sup>

It seems that the mass of basal roots produced by a piece of defoliated stem also increases with the mass of the stem.

<sup>5</sup> A plant morphologist, to whom the writer showed these experiments, commented that he was convinced that the shoot formation of an isolated piece of stem was due to a "stimulus." If we accept this suggestion, it follows that the "stimulus" for regeneration must have varied quantitatively with the mass of the defoliated stem in our experiments, and this would lead us again to the idea that the "stimulus" must be something material since it cannot well be spiritual.





# ELECTRICAL CHARGES OF COLLOIDAL PARTICLES AND ANOMALOUS OSMOSIS.

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## *I. The Transport Curves on the Acid Side of the Isoelectric Point of the Membrane.*

The experiments described in a preceding paper<sup>1</sup> leave no doubt that the Donnan equilibrium is the main source of the potential differences between solid gelatin particles and the surrounding liquid. On the other hand, experiments on the influence of salts on electrical endosmose, cataphoresis, anomalous osmosis, and Quincke's current potentials suggest in certain cases at least a second source which is generally designated as adsorption potentials. The difference between the two kinds of potentials should be that while the potential differences due to the Donnan equilibrium depend on the ionization of the protein, the adsorption potentials should occur regardless of whether or not the solid colloid is ionized. Adsorption potentials should, therefore, be found just as well in the case of isoelectric protein where the protein is practically non-ionized as in the case of metal proteinates or protein-acid salts, while the p.d. due to the Donnan equilibrium should be restricted to the latter two forms of protein.<sup>2</sup>

It is intended to investigate on the basis of this idea whether or not there exist at the surface of solid gelatin adsorption potentials in addition to potentials due to the Donnan equilibrium. It will be necessary to use for this purpose either electrical endosmose or anomalous osmosis or Quincke's current potentials or cataphoresis. We shall select in this paper anomalous osmosis. By anomalous osmosis

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 351.

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 667; 1921-22, iv, 351.

is meant the superposition of electrical forces over the purely osmotic forces in the transport of water through a membrane separating pure water from a solution of an electrolyte (or separating two different solutions of electrolytes). When both water and electrolytes are capable of diffusing through the membrane the difference in the mobility of the oppositely charged ions will cause diffusion potentials acting across the membrane. In this case the solution assumes the opposite sign of charge as the water. These potentials we will call  $E$ .

There may be a second P.D. inside the pores of the membrane between the solid wall of the pore and the liquid inside the pore. This potential we will call  $\epsilon$ . If as the consequence of  $\epsilon$  the liquid inside the pore assumes a negative charge, while as a consequence of  $E$  the solution in the collodion bag assumes a positive charge, the liquid cylinder inside the pore will be dragged into the solution by these electrical forces and thus an electrical transport of water will be added to the transport of water by osmotic forces. If the solution, however, has the same sign of charge as the liquid inside the pore, the electrical force will act in an opposite sense from the osmotic force, and the flow of water from the water side of the membrane into the solution will be less than is to be expected on the basis of van't Hoff's law.

This theory of anomalous osmosis was first suggested by Girard<sup>3</sup> and has later been supported by Bartell<sup>4</sup> and others.

In the experiments to be described salt solutions of a definite pH but of different concentrations were put into collodion flasks of about 50 cc. volume, which had received a coating of gelatin as described in previous papers. The collodion bags were dipped into 350 cc. of water of the same pH as that of the salt solution, but containing no salt. The collodion bags were closed with a rubber stopper perforated by a glass tube serving as manometer. The temperature was 24°C. and the rise of the liquid in the manometer was read 20 minutes after the commencement of each experiment. In the close regulation of

<sup>3</sup> Girard, P., *Compt. rend. Acad.*, 1908, cxlvi, 927; 1909, cxlviii, 1047, 1186; 1910, cl, 1446; 1911, cliii, 401. Girard, P., *La pression osmotique et le mécanisme de l'osmose*, Publications de la Société de Chimie-physique, Paris, 1912.

<sup>4</sup> Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036. Bartell, F. E., and Madison, O. E., *J. Physical Chem.*, 1920, xxiv, 593.

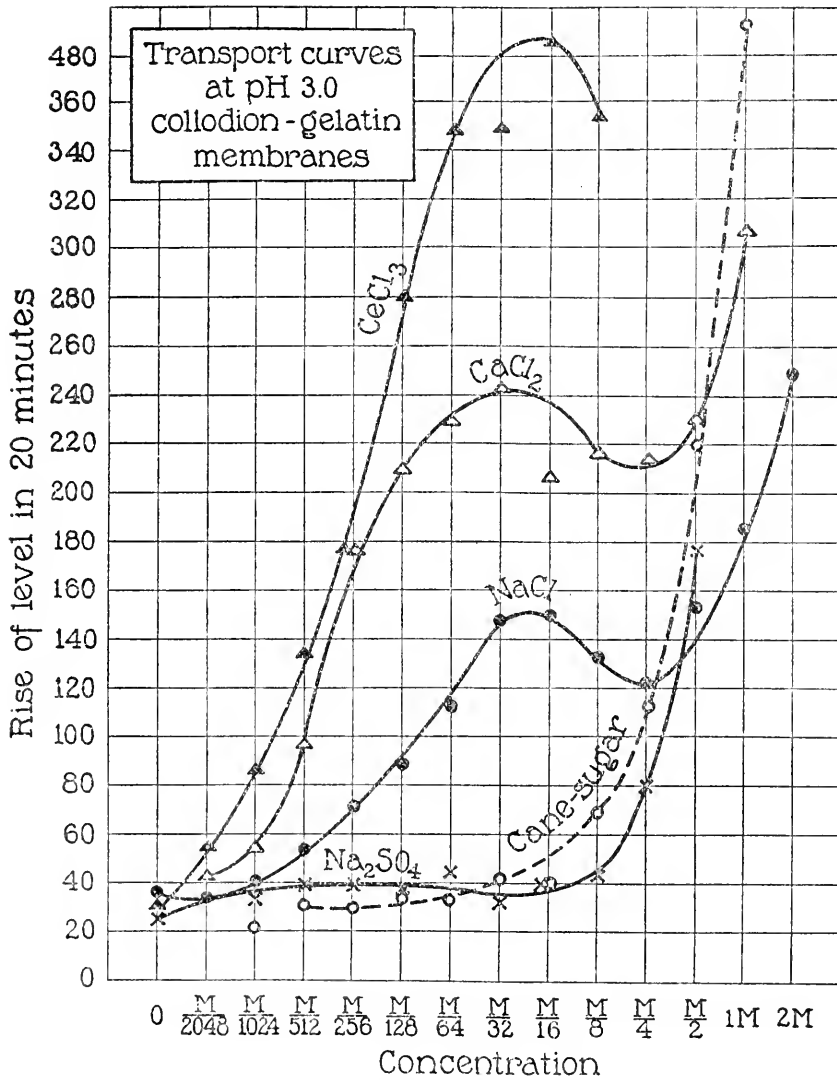


FIG. 1. Transport curves of liquid from the side of water to the side of salt solution through collodion-gelatin membranes of pH 3.0. Abscissæ are the concentration of salt, ordinates the rise in mm. of level in manometer in solution after 20 minutes. Notice the difference between the transport curve for cane-sugar and those for  $\text{NaCl}$ ,  $\text{CaCl}_2$ , and  $\text{CeCl}_3$ .

the hydrogen ion concentration the experiments to be given in this paper differ from those of previous workers.

Fig. 1 represents the transport curves for different concentrations of  $\text{CeCl}_3$ ,  $\text{CaCl}_2$ ,  $\text{NaCl}$ ,  $\text{Na}_2\text{SO}_4$ , and cane-sugar, all of pH 3.0 (HCl having been added). The outside solution was an HCl solution also of pH 3.0 (approximately  $N/1,000$  HCl), but containing no salt. The abscissæ are the concentrations, the ordinates the level in mm. to which the liquid had risen in the manometer after 20 minutes. The results of these experiments corroborate similar experiments already published.<sup>5</sup>

The curves for the first three salts,  $\text{CeCl}_3$ ,  $\text{CaCl}_2$ , and  $\text{NaCl}$ , rise at first until the concentration is about  $M/32$ , then fall and then rise again at a concentration of about  $M/4$ . The curves for cane-sugar and  $\text{Na}_2\text{SO}_4$  commence to rise at a concentration of about  $M/32$  or  $M/8$  respectively. The transport of water therefore increases with the valency of the cation and inversely with the valency of the anion and the question arises how to account for these curves.

According to Helmholtz's formula modified by Perrin, the transport of liquid through a capillary under the influence of a direct current is

$$v = \frac{q \cdot \epsilon \cdot E \cdot D}{4 \pi \cdot \eta \cdot L}$$

where  $v$  is the quantity of liquid carried electroosmotically,  $q$  the cross section of the capillary,  $\epsilon$  is the potential difference between the two strata of the double layer inside the capillary,  $E$  the external electromotive force (acting at right angles to the electrical double layer in the capillary),  $D$  the dielectric constant of the medium,  $\eta$  the coefficient of internal friction, and  $L$  the distance of the external electrodes.

In the experiments on anomalous osmosis the driving force,  $E$ , is not furnished by an external P.D. but by a P.D. across the membrane, which has its origin in the difference between the solutions on the opposite sides of the membrane and which acts only through the extremely small distance of the thickness of the membrane.  $\epsilon$  is the P.D. between the liquid inside the pores and the wall of the gelatin film. At pH 3.0, gelatin exists in the form of gelatin-acid salts, e.g. gelatin

<sup>5</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 387, 563, 577, 659, 673.

chloride, when the acid is HCl. The water inside the pores of a film of gelatin chloride is negatively charged while the gelatin is positively charged. This is a consequence of the theory of Donnan's membrane equilibrium.<sup>1</sup>

In these experiments the gelatin with which the membrane was coated was originally isoelectric, but the film of gelatin was brought into equilibrium with water of pH 3.0 by putting the collodion bags for several hours into HCl of pH 3.0 before the beginning of the experiment. Since the addition of salts influences both the P.D. across the membrane (*i.e.* the value of  $E$ ) as well as the P.D. between the liquid inside the pores and the wall of the pore (*i.e.* the value of  $\epsilon$ ) it is necessary to measure the influence of salts on these two P.D. separately and then try to use the results for the analysis of the curves in Fig. 1. The measurements of the P.D. across the membrane were made with the aid of a Compton electrometer and the two electrodes used were calomel electrodes with saturated KCl solution. Measurements of the P.D. across the membrane were made at the beginning of the experiment (Table I) and at the end; *i.e.*, after 20 minutes (Table II). The figures for the P.D. in Table II are lower than in Table I for the reason that during the experiment part of the salt diffused into the outside solution so that the concentration of the salt solution inside the bag diminished while that in the outside increased; hence the value of  $E$  diminished. In addition the hydrogen ion concentration which was the same inside and outside the salt solution at the beginning changed and this added a complication which has been discussed in a previous paper.<sup>6</sup> Table I shows that the P.D. across the membrane ( $E$ ) increases with the valency of the cation and inversely with the valency of the anion,  $E$  being a maximum for  $\text{CeCl}_3$ , being lower for  $\text{CaCl}_2$ , and still lower for  $\text{NaCl}$ ; for  $\text{Na}_2\text{SO}_4$  it becomes about zero or even slightly negative (Table II). It is, therefore, obvious that in a semiquantitative way the results of Tables I and II (*i.e.* the values of  $E$ ) explain the difference in the ascending branches of the curves in Fig. 1 up to a concentration of about  $M/32$ .

It can be shown that this P.D. is at least partly due to diffusion potentials. In measuring the diffusion potentials the principle of a

<sup>6</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 213.

TABLE I.

*Influence of Concentration of Salt on the Value of E.*

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against H<sub>2</sub>O of pH 3.0 (acid used, HCl), at beginning of experiment. Sign of charge of solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
CeCl <sub>3</sub> .....	1.0	9.0	15.0	21.0	32.0	41.0	45.0	41.0	41.0	41.5				
CaCl <sub>2</sub> .....	0.0	8.0	16.0	16.0	23.0	25.0	35.0	34.0	33.0	39.0	42.5	47.0	49.0	
NaCl.....	1.5	5.5	8.0	12.0	13.0	18.0	22.0	20.0	23.0	23.0	24.0	25.0	28.0	28.0
Na <sub>2</sub> SO <sub>4</sub> .....	0.5	5.0	6.0	5.0	8.5	7.5	5.0	-1.5	-2.0	-8.0	-12.0	-17.5		
Cane-sugar.....			2.0	2.0	0	0	0	-1.0	0	0	-0.5	1.0	0	

TABLE II.

*Influence of Concentration of Salt on the Value of E.*

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against H<sub>2</sub>O of pH 3.0 (acid used, HCl), after 20 minutes from beginning of experiment. Sign of charge of solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
CeCl <sub>3</sub> .....	-0.5	10.0	16.0	22.5	28.0	35.0	41.0	32.0	32.0	29.0				
CaCl <sub>2</sub> .....	-1.0	6.5	11.5	17.0	22.0	23.0	30.0	27.5	25.0	25.0	24.0	23.0	20.0	
NaCl.....	1.5	4.0	7.5	12.0	10.0	13.0	16.5	16.0	16.0	15.0	11.0	12.0	10.5	8.0
Na <sub>2</sub> SO <sub>4</sub> .....	0	3.5	1.0	1.0	1.0	-0.5	-2.5	-6.0	-6.0	-10.0	-10.0	-13.0		
Cane-sugar.....			3.0	6.0	2.0	2.0	3.0	4.0	0	1.0	3.0	5.0	1.0	

flowing junction of Lamb and Larson<sup>7</sup> was used in a simplified and also less accurate form, which, however, gave results of sufficient accuracy for our purpose. Table III gives some of the results showing that the order of efficiency of the various salts and the influence of concentration are the same in diffusion potentials as in the p.d. across the membrane. The diffusion potentials are, however, considerably lower than the p.d. across the membrane (Tables I and II). The diffusion potentials depend on the difference in the relative velocity of the oppositely charged ions of a salt. A comparison of the values in Table I and Table III suggests that the source of the p.d. across the membrane is the same as that of the diffusion potential if we assume that at pH 3.0 the cations experience a greater retardation in the diffusion through protein films than anions.

The figures in Table I, II, or III do, however, not explain the drop in the curves of Fig. 1 which occurs when the concentration of the salt reaches  $m/32$ . The cause for this depression lies probably in the influence of the concentration of the salt on the value of  $\epsilon$ ; *i.e.*, p.d. between gelatin chloride and water inside the pores of the membrane. It has been shown in previous experiments that salts depress the p.d. between gelatin particles and surrounding liquid and that the reason for this depression is furnished by the Donnan theory of membrane equilibria.<sup>1</sup> The method of these experiments was briefly as follows:

1 gm. of fine particles of powdered gelatin rendered first isoelectric and of an equal size of grain was put into 200 cc. of various concentrations of a salt ( $\text{NaCl}$ ,  $\text{CaCl}_2$ , etc.) in water and containing 8 cc. of 0.1 N  $\text{HCl}$  per 100 cc. The gelatin remained in this solution for 2 hours at 20°C. under frequent stirring. The suspension was then put on a filter and the gelatin freed from the supernatant liquid. The gelatin was then melted by heating to 45° and cooled rapidly to cause solidification and the p.d. between the gelatin and the supernatant liquid was then measured with the aid of a Compton electrometer. Table IV gives the result. The reader will notice that in no case do any of the salts cause a rise in the p.d. between gelatin and liquid. The observed p.d. could, however, be calculated with a fair degree of accuracy from Donnan's equilibrium equation.

<sup>7</sup> Lamb, A. B., and Larson, A. T., *J. Am. Chem. Soc.*, 1920, xlii, 229.

TABLE III.

Diffusion potentials of different concentrations of salts of pH 3.0 against H<sub>2</sub>O of pH 3.0 (acid used, HCl). Sign of charge of salt solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
CeCl <sub>3</sub> .....		2.0	4.0	7.0	10.0	16.0	19.0	24.0	31.0	36.0			
CaCl <sub>2</sub> .....		2.0	3.0	6.0	10.0	13.0	17.0	22.0	26.0	30.0	35.0	43.0	47.0
NaCl.....	0	0	0.5	1.5	3.0	5.0	7.0	9.0	12.0	15.0	15.0	17.0	18.0
Na <sub>2</sub> SO <sub>4</sub> .....		-1.0	-1.0	-1.5	-2.0	-3.0	-5.0	-7.0	-9.0	-12.0	-15.0	-22.0	
Cane-sugar.....		0	0	0	0	0	-0.5	-0.5	-0.5	-1.0	-1.5	-3.0	-3.0

TABLE IV.

*Influence of Different Concentrations of Salts on the Value of  $\epsilon$ .*  
p.d. in millivolts between solid gelatin particles and HCl solution. pH of gelatin particles about 2.8 (in absence of salt).

Concentration.	0	M/8192	M/4096	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
NaCl.....	25.5	23.0	25.0	25.0	23.0	21.5	18.5	14.0	10.5	7.0	5.5	2.5
CaCl <sub>2</sub> .....	25.5	25.5	23.0	24.0	21.0	19.0	15.5	11.5	7.5	5.0	3.0	2.5
BaCl <sub>2</sub> .....	26.0	25.0	24.0	23.0	22.0	18.5	15.0	11.0	7.5	5.5	2.5	2.0
CeCl <sub>3</sub> .....	26.0	25.0	22.0	21.5	19.0	16.0	11.5	8.0	5.0	2.5	2.5	
Na <sub>2</sub> SO <sub>4</sub> .....	25.0	22.0	22.5	21.0	18.0	16.0	11.5	8.5	6.5	4.0	3.0	1.5



The effect of the salt on  $\epsilon$  was depressing and no increase in  $\epsilon$  is noticeable in the low concentrations of  $\text{CeCl}_3$  or  $\text{CaCl}_2$ . It is therefore obvious that the effect of the salts on  $\epsilon$  exhibited by our method of experimenting can account for the drop in the curves in Fig. 1 but apparently not for the initial rise or the augmenting effect of low concentrations of  $\text{CeCl}_3$  or  $\text{CaCl}_2$ . If Helmholtz's formula holds for these experiments curves representing the product of the values  $E \times \epsilon$  should therefore show an initial rise, followed by a drop; moreover, they should show the relative order of the rise as exhibited in the transport curves in Fig. 1.

In Fig. 2 the curves for  $E \times \epsilon$  are plotted with the concentration of the salt as abscissæ and the value of  $E \times \epsilon$  as ordinates. The values of  $\epsilon$  are taken from Table IV and those for  $E$  from Table II. The general order of the four curves in Fig. 2 is sufficiently similar to that in Fig. 1 to indicate that our interpretation of the curves in Fig. 1 is approximately correct. Thus the transport curve for  $\text{Na}_2\text{SO}_4$  in Fig. 1 is flat, and so is the  $E \times \epsilon$  curve for  $\text{Na}_2\text{SO}_4$  in Fig. 2. The curves for  $\text{NaCl}$ ,  $\text{CaCl}_2$ , and  $\text{CeCl}_3$  rise in both figures in the order named and in both curves the rise is followed by a drop. The second rise of the curves in Fig. 1 after a concentration of  $M/4$  is due to the osmotic effect and has no connection with the electrical effect, as is shown by the fact that this rise occurs also in the cane-sugar curve. Hence, as far as the electrical effect in Fig. 1 is concerned, the character of the curves resembles that of the curves in Fig. 2, as is proven by the fact that if we superpose the curves for  $E \times \epsilon$  in Fig. 2 over the transport curve for cane-sugar in Fig. 1 we get the curves of the type of Fig. 1. This is still more approximately the case if we substitute for the purely osmotic transport effect not the curve for cane-sugar but the curves for  $\text{NaCl}$  and  $\text{CaCl}_2$  at the isoelectric point of gelatin (Fig. 4). Since the curve for  $\text{NaCl}$  at pH 4.7 (Fig. 4) rises more slowly than the corresponding curve for  $\text{CaCl}_2$ , the lowest point in the  $\text{NaCl}$  curve in Fig. 1 must be lower than that for  $\text{CaCl}_2$ .

This explains the empirical rule at which the writer had arrived in his previous papers<sup>5</sup> on anomalous osmosis; namely, first, that the transport of water through the collodion-gelatin membrane from the side of the water to the side of solution is increased by that ion of the salt which has the same sign of charge as the membrane, and dimin-

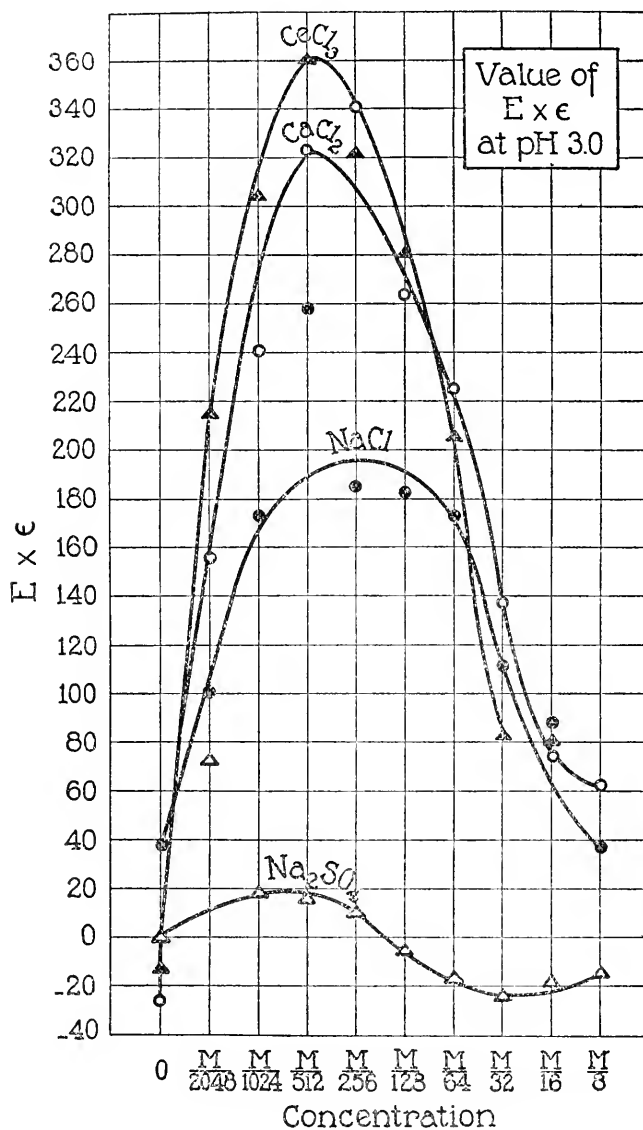


FIG. 2. Value of product of p.d. across the membrane ( $E$ ) and p.d. inside the pores of the membrane ( $\epsilon$ ) at pH 3.0. Abscissæ are the concentration of salt solution, ordinates  $E \times \epsilon$ . Notice similarity of curves for  $E \times \epsilon$  with the curves in Fig. 1 at lower concentrations of salt.

ished by the oppositely charged ion with a force increasing with the valency of the ion; and second, that the relative effect of the oppositely charged ions is not the same at different concentrations, but that at low concentrations the augmenting effect of the ion with the same sign of charge as the membrane increases more rapidly than the depressing effect of the oppositely charged ion, while at high concentrations the reverse is true. The augmenting effect of the ion with the same sign of charge as that of the membrane on the transport of liquid is due chiefly, if not exclusively, to the effect of the salt on the p.d. across the membrane ( $E$ ), which depends upon that ion which gives the salt solution the opposite charge from that of the liquid inside the pores. This ion is in this case the cation and hence the transport increases with the valency of the cation (Tables I and II). The depressing effect of the oppositely charged ion on the transport of liquid is due to the effect on  $\epsilon$ . If  $\epsilon$  is determined by the Donnan equilibrium between the solid gelatin salt and the bounding solution, it must, according to the theory, be depressed by that ion which has the opposite sign of charge as the protein ion.<sup>1</sup> The oppositely charged ions of a salt act, therefore, each on a different type of p.d., the one on diffusion potentials, the other on a p.d. due to an equilibrium condition.

There are three discrepancies between the curves in Figs. 1 and 2 which need further explanation. First, the location of the maximum of the curves in Figs. 1 and 2 is not identical, being located at  $M/32$  in Fig. 1 and at  $M/256$  or  $M/512$  in Fig. 2. This may be partly or entirely due to the fact that the concentration of the liquid was lower inside the pores than in the salt solution since water was flowing constantly from the side of water into the solution, thus causing a considerable dilution inside the pores.

Second, the curves in Fig. 1 do not come down to zero while those in Fig. 2 come down to nearly zero at a nominal concentration of  $M/8$ . For this we may have two reasons, first, that when the concentration exceeds  $M/4$  the transport due to osmotic forces becomes so great that a drop of the transport curves to zero is no longer possible; or it may mean that after the concentration exceeds  $M/4$  a new source of electrification of the gelatin inside the pores not accounted for by the ionization of the protein commences. We shall return to this

possibility later and show that there is no adequate support for this second assumption, though it cannot be absolutely excluded.

Third, the difference between the curves for  $\text{CeCl}_3$  and  $\text{CaCl}_2$  is smaller in Fig. 2 than in Fig. 1. It is possible that Ce increases the value of  $\epsilon$  beyond that accounted for by the ionization of gelatin chloride.

Aside from these discrepancies we can say that Helmholtz's formula explains the curves for anomalous osmosis given in Fig. 1 when the values for  $\epsilon$  used are those to be expected on the basis of the Donnan equilibrium. It may, therefore, be stated that the Donnan theory is able to explain the phenomena of anomalous osmosis more completely than any other theory thus far offered.

## *II. The Transport Curves on the Alkaline Side of the Isoelectric Point.*

In these experiments the salt solutions were rendered alkaline by adding enough KOH to bring the salt solution to a pH of 11.0. The outside solution was a pure KOH solution also of pH 11.0 but free from salt. Fig. 3 gives the curves for the transport of liquid in the solution during the first 20 minutes. The curves show a rise—until the concentration of the salt is about  $M/64$ —followed by a drop, and then a second rise follows at a concentration of about  $M/8$ . The general character of the curves in Fig. 3 is about the same as that in Fig. 1 but the relative efficiency of the cations and anions is reversed. In solutions whose pH is on the alkaline side of the isoelectric point of gelatin, the "attraction" of the solution for water increases with the valency of the anion but inversely with the valency of the cation; while on the acid side the relative efficiency of the two oppositely charged ions is the reverse. Thus in Fig. 1 the curve for  $\text{Na}_2\text{SO}_4$  is flat while that for  $\text{CaCl}_2$  rises; in Fig. 3 the curve for  $\text{Na}_2\text{SO}_4$  rises while that for  $\text{CaCl}_2$  is flat. The reason for this reversal is the fact that the sign of charge between the liquid inside the pores of the gelatin film and the gelatin wall of the pore is reversed on the opposite sides of the isoelectric point. At pH 3.0 the gelatin is positively charged and the liquid inside the pores is negatively charged; while at pH 11.0 the gelatin is negatively charged and the liquid inside the pores is positively charged. The sign of charge of the solution in the P.D. across the membrane, *i.e.* of  $E$ , remains, however, the same in alkali and acid solutions (Table V).

Table V gives the p.d. across the membrane at the beginning of the experiments represented in Fig. 3 and Table VI gives the diffusion potentials between the same salt solutions of pH 11.0 against KOH of pH 11.0 without salts and with no membrane between them.

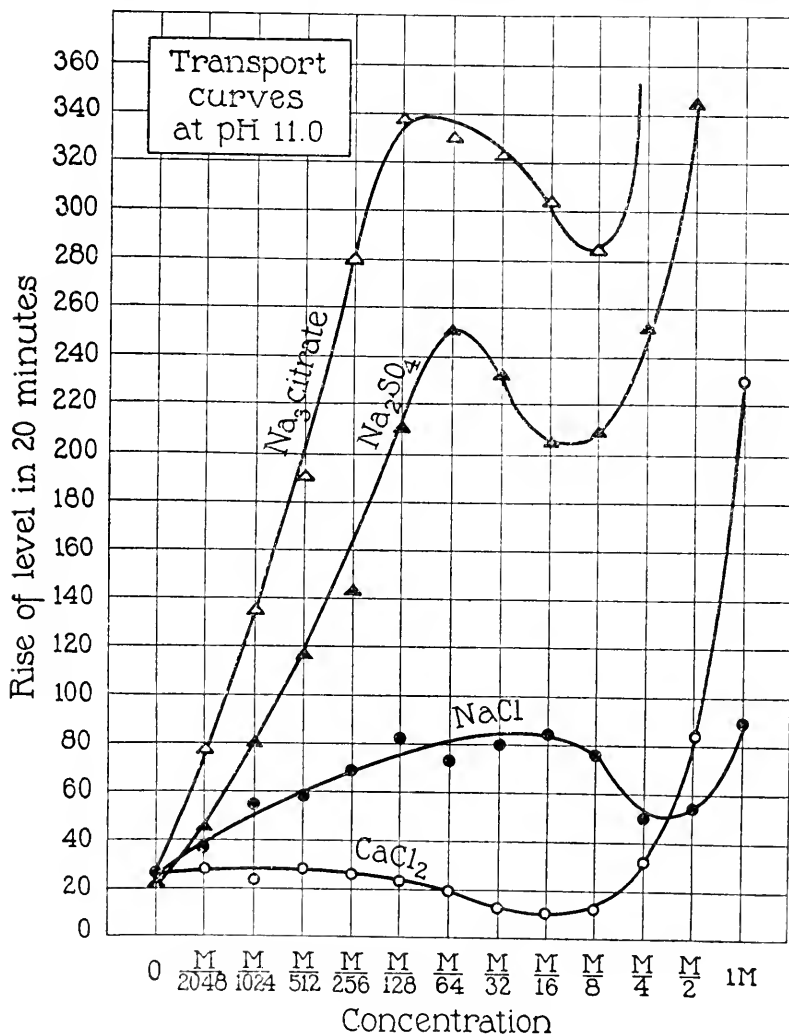


FIG. 3. Transport curves at pH 11.0. Notice reversal of relative efficiency of anions and cations between the curves in Figs. 1 and 3, due to the fact that the sign of charge of liquid inside the pores is positive at pH 3.0 and negative at pH 11.0.

TABLE V.

p.D. across a collodion-gelatin membrane between different concentrations of salts of pH 11.0 against H<sub>2</sub>O of pH 11.0 (alkali used, KOH) at beginning of diffusion. Sign of charge of salt solution always positive unless minus sign is added.

Concentration.	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
Na <sub>3</sub> citrate.....	-20.0	-27.0	-38.0	-34.0	-37.0	-39.0	-33.5	-37.0	-44.0	-46.0	-52.0	-56.0
Na <sub>2</sub> SO <sub>4</sub> .....	-15.0	-23.0	-25.0	-28.0	-29.0	-25.0	-23.0	-21.0	-23.0	-26.5	-30.0	
NaCl.....	-5.0	-8.0	-13.0	-7.0	-10.0	1.0	4.5	9.5	13.5	19.0	22.0	24.0
CaCl <sub>2</sub> .....	-4.0	-2.5	0	6.5	14.0	20.0	26.5	33.0	38.0	42.5	49.0	50.0

TABLE VI.

Diffusion potentials in millivolts of different concentrations of salts of pH 11.0 against H<sub>2</sub>O of pH 11.0 (alkali used, KOH.) Sign of charge of salt solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
Na <sub>3</sub> citrate.....	0	-4.0	-6.0	-9.0	-12.0	-15.5	-20.0	-24.0	-28.5	-33.5	-38.0	-43.0
Na <sub>2</sub> SO <sub>4</sub> .....	0	-2.5	-3.0	-4.0	-5.0	-6.5	-8.5	-11.0	-14.0	-17.0	-19.5	-22.0
NaCl.....	0	1.0	1.5	3.0	5.0	8.0	10.0	12.5	15.0	18.0	20.5	23.5
CaCl <sub>2</sub> .....	0	4.5	7.0	10.5	15.0	19.5	23.5	28.5	34.0	39.0	43.0	48.0

We can say that the sign and order of the values of the potentials are the same in Tables V and VI indicating that the p.d. across the membrane is essentially of the nature of a diffusion potential. The negative p.d. across the membrane in Table V is, however, always greater than the corresponding p.d. across the membrane in Table VI.

The drop in the curves in Fig. 3 beyond a concentration of  $M/64$  is due again to the diminution of  $\epsilon$  through the increase in the concentration of salts.

### *III. The Transport Curves at the Isoelectric Point.*

The main purpose of this paper is the investigation of the transport curves at the isoelectric point. At this point the gelatin is not ionized and salts cannot cause a charge of the particles unless they alter the pH or cause the formation of complex protein salts which undergo an electrolytic dissociation. This latter seems to occur when salts with trivalent (or tetravalent?) cations or salts with tetravalent anions are added to isoelectric gelatin, since the addition of this kind of salts has a similar effect as the addition of acid or alkali respectively to isoelectric gelatin. No such effect seems, however, noticeable in the case of salts of the type of NaCl, CaCl<sub>2</sub>, or Na<sub>2</sub>SO<sub>4</sub>. These latter salts influence the transport curves at the isoelectric point in approximately the same way as does cane-sugar or grape sugar; *i.e.*, only osmotically. The transport curves for these latter salts show no electrical effect at the isoelectric point but only the osmotic effect (Fig. 4). In these experiments at the isoelectric point special care was taken that the gelatin film of the membrane was at the isoelectric point at the beginning of the experiment; *i.e.*, that the pH was 4.7. The gelatin used for the film formation was isoelectric and in addition the collodion-gelatin bags were kept in water which had been brought to pH 4.7, by adding acetic acid. The salt solutions also were carefully brought to pH 4.7.

Table VII shows that the p.d. across the membrane is very high at pH 4.7.<sup>8</sup> If, therefore, at this pH there exists only a small p.d.

<sup>8</sup> If we compare the p.d. across the membrane for the NaCl and CaCl<sub>2</sub> and CeCl<sub>3</sub> solutions with the diffusion potentials (which are not given in this paper), it is seen that the p.d. across the membrane is greater than the diffusion potentials for the same solutions, as if the membrane retarded the motion of the cations. The same was found when the pH was 3.0. It is, therefore, obvious that this cation retention is not caused by the charge of the gelatin.

TABLE VII.

p.d. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 4.7 (isoelectric point of gelatin) against H<sub>2</sub>O of pH 4.7, at beginning of experiment. Sign of charge of solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
KCl.....		1.0	0.5	- 1.0	- 3.5	- 2.5	- 1.0	- 1.0	- 0.5	0	0	0	0	0
NaCl.....	4.0	9.0	10.0	13.0	15.0	19.0	22.0	25.0	28.0	27.0	28.0	33.0	34.0	
LiCl.....	0	13.0	14.0	18.0	26.0	32.0	38.0	36.0	39.0	49.0	51.0	57.0	55.0	59.0
MgCl <sub>2</sub> .....	4.0	20.0	25.0	35.0	81.0	48.0	52.0	55.0	62.5	64.0	74.0	72.5	75.0	
CaCl <sub>2</sub> .....	5.0	27.0	31.5	33.0	39.0	45.0	44.0	52.0	54.0	62.5	68.0	70.0	71.0	
BaCl <sub>2</sub> .....	3.0	14.0	23.0	25.0	27.5	33.0	43.0	48.0	52.0	57.0	57.0	61.0	64.0	
MgSO <sub>4</sub> .....	1.5	1.0	4.0	6.0	8.0	10.0	11.0	10.0	9.0	10.0	9.5	9.0	5.0	
Ce(NO <sub>3</sub> ) <sub>3</sub> .....	0	17.0	28.0	41.0	44.0	49.0	50.0	54.0	55.0					
Na <sub>2</sub> SO <sub>4</sub> .....	4.0	- 4.5	- 7.0	- 8.0	- 11.0	- 14.0	- 16.0	- 18.5	- 20.0	- 25.0	- 28.0	- 32.5		
Na <sub>4</sub> Fe(CN).....		- 28.0	- 30.0	- 30.0	- 31.0	- 33.0	- 35.0	- 37.5	- 45.0	- 50.0	- 60.0			
Cane-sugar.....	3.0	2.5	5.0	2.5	4.0	3.0	3.5	3.0	2.5	2.5	3.0	- 1.0	- 1.5	



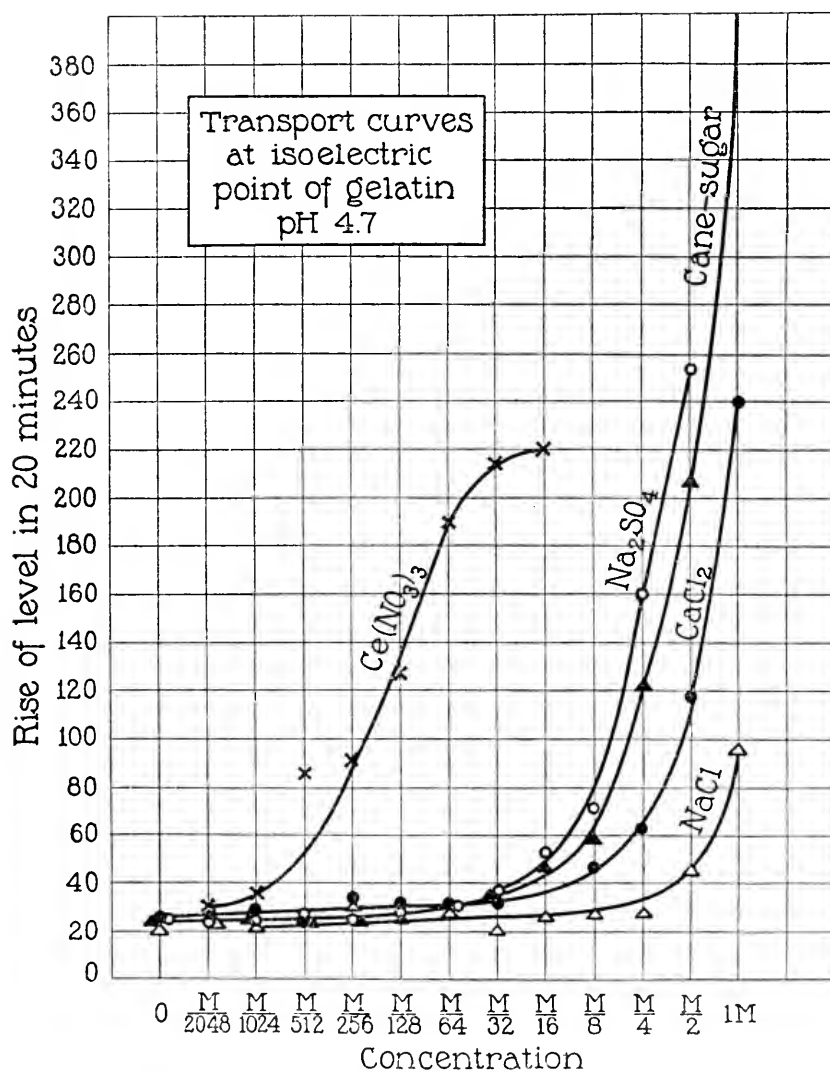


FIG. 4. Transport curves at the isoelectric point of gelatin. The curves for  $NaCl$ ,  $CaCl_2$ , and  $Na_2SO_4$  resemble the transport curve for cane-sugar showing that osmotic forces suffice to explain these curves. The curve for  $Ce(NO_3)_3$  suggests that this salt confers a positive charge to the gelatin.

inside the pores of the membrane a considerable transport of water by electrical forces must occur. If no such transport is noticeable, it means that  $\epsilon$  is zero (as the Donnan theory demands, since gelatin

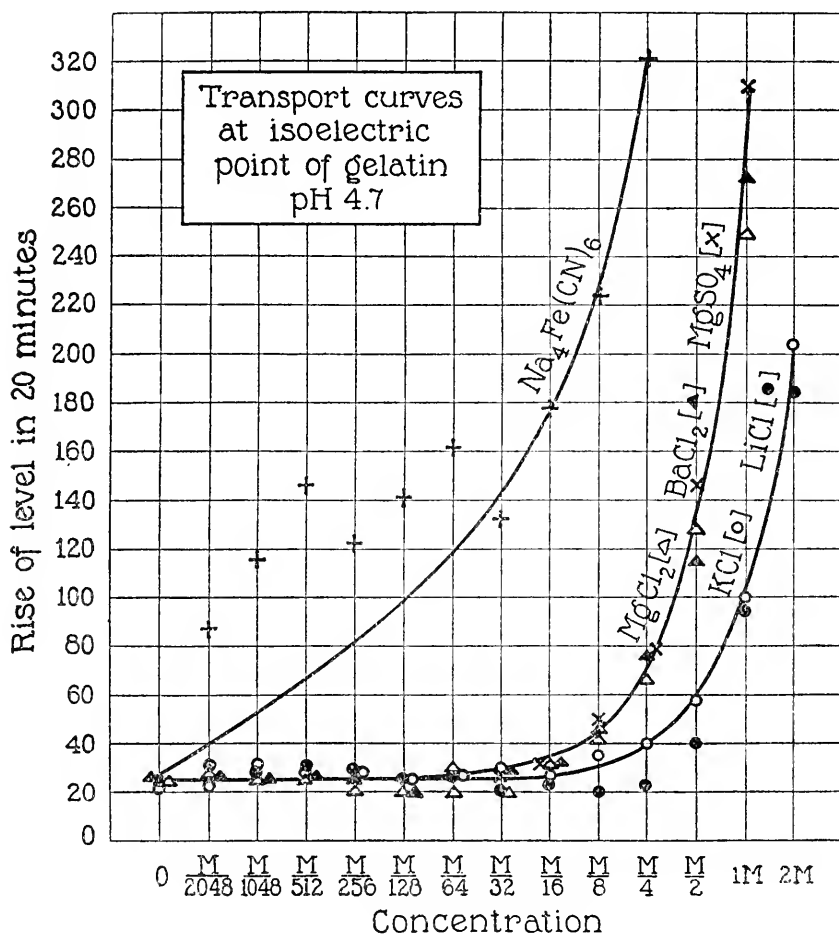


FIG. 5. Transport curves at the isoelectric point. Those for KCl, LiCl,  $\text{MgCl}_2$ ,  $\text{BaCl}_2$ , and  $\text{MgSO}_4$  resemble the transport curves for cane-sugar while  $\text{Na}_4\text{Fe}(\text{CN})_6$  seems to transfer a negative charge to the isoelectric gelatin.

is not ionized at its isoelectric point and does not combine with salt), and that there is no additional source of P.D. which might be ascribed to adsorption. Figs. 4 and 5 give the transport curves for cane-sugar, NaCl, KCl, LiCl,  $\text{MgCl}_2$ ,  $\text{MgSO}_4$ ,  $\text{BaCl}_2$ ,  $\text{Ce}(\text{NO}_3)_3$ , and  $\text{Na}_4\text{Fe}(\text{CN})_6$  at pH 4.7; *i.e.*, when gelatin is non-ionized. There cannot be the least doubt that the curves for all these salts (with the exception of  $\text{Ce}(\text{NO}_3)_3$  and  $\text{Na}_4\text{Fe}(\text{CN})_6$ ) are of the nature of the cane-sugar curve; *i.e.*, they show only that part of the curve which corresponds to the second rise of the transport curves in Figs. 1 and 3, and which must be ascribed chiefly, if not exclusively, to the osmotic forces. It might be added that the curve for  $\text{Na}_2$  oxalate does not commence to rise until the concentration of the salt is  $M/16$ . The initial rise and drop of the transport curves in Figs. 1 and 3, which is the expression of the electrical forces, is entirely lacking in all the curves at the isoelectric point of gelatin, Figs. 4 and 5, with the exception of the curves for  $\text{Ce}(\text{NO}_3)_3$  and  $\text{Na}_4\text{Fe}(\text{CN})_6$ , to which we shall now give our attention.

In previous publications the writer has already called attention to the fact that on the alkaline side of the isoelectric point the presence of salts with a trivalent cation has the effect of reversing the sign of the P.D. between gelatin and water. When the pH is  $> 4.7$ , *i.e.* when gelatin exists in the form of Na gelatinate, the Donnan equilibrium causes the expulsion of NaOH from the gelatin into the bounding liquid with the result that gelatin assumes a negative and the bounding liquid a positive charge. When, however, some  $\text{CeCl}_3$  or  $\text{LaCl}_3$  is added the liquid assumes a negative and the gelatin a positive charge.<sup>9</sup> This reversal of the sign of charge by trivalent cations had been discovered by Perrin in his experiments on electrical endosmose.<sup>10</sup> The reversal may either be due to a reaction between  $\text{Ce}(\text{NO}_3)_3$  and isoelectric gelatin, in which a compound is formed which dissociates into a complex positively charged gelatin-Ce cation and a negative ion, presumably  $\text{NO}_3$ , or the addition of the salt brings the pH to a value below 4.7. In either case the behavior of the curve for  $\text{Ce}(\text{NO}_3)_3$  in Fig. 4 becomes clear. When  $\text{Ce}(\text{NO}_3)_3$  solutions of pH 4.7 are separated by a collodion-gelatin membrane (of

<sup>9</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 659.

<sup>10</sup> Perrin, J., *J. chim. physique*, 1904, ii, 601; 1905, iii, 50. Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918.

pH 4.7) from water of pH 4.7, the  $\text{Ce}(\text{NO}_3)_3$  solution assumes a positive charge as is shown in Table VII. If now the Ce causes the liquid cylinders inside the pores to be charged negatively an electrical transport of water into the solution must occur commencing at a low concentration of the salt and in the way characteristic for the electrical transport curves in Figs. 1 and 3. The curve for  $\text{Na}_4\text{Fe}(\text{CN})_6$  in Fig. 5 suggests that  $\text{Na}_4\text{Fe}(\text{CN})_6$  causes solid isoelectric gelatin to assume a negative charge. The question is, whether this happens only in the case of trivalent (and probably tetravalent) cations and tetravalent anions. It is obvious that all the transport curves for salts with divalent cations, Mg, Ca, and Ba, in Figs. 4 and 5, commence to rise at a slightly lower concentration than the transport curves for the salts with monovalent cation, KCl, NaCl, and LiCl. It might be argued that the salts with bivalent cation transfer also a positive charge to isoelectric gelatin at a concentration above  $M/8$  and that the same is true for the salts with monovalent cations, the difference being that the concentration of the salt required for this effect is very high for salts with monovalent cation, slightly lower for salts with bivalent cation, and very low for salts with trivalent cation.

On the other hand it should be pointed out that the transport curve for cane-sugar rises even more rapidly than that for  $\text{CaCl}_2$  so that there is in reality no need to assume that the  $\text{CaCl}_2$  charges solid isoelectric gelatin positively. The difference between the transport curves for NaCl and  $\text{CaCl}_2$  in Fig. 4 is no greater than the difference between the transport curves for cane-sugar and grape sugar. As far as the experiments on anomalous osmosis are concerned, there is no reason to assume that the salts with divalent cations or monovalent cations transfer a positive charge to the isoelectric gelatin or to gelatin of any pH or that the bivalent anions transfer a negative charge, though it is not absolutely disproved that this may not happen at high concentrations of the salts.

It was thus far left undecided whether the positive electrification of isoelectric gelatin by  $\text{Ce}(\text{NO}_3)_3$  in Fig. 4 was due to a change of the pH so that the gelatin was no longer isoelectric or to the formation of a salt between isoelectric gelatin and  $\text{Ce}(\text{NO}_3)_3$  dissociating into a positively charged complex gelatin-Ce ion and negatively charged  $\text{NO}_3$  ions. To settle this question experiments were made with

buffer solutions consisting of mixtures of  $M/50$  acetic acid and  $M/50$  Na acetate in the proper proportions to give a pH 4.7, 5.6, or 3.4. Solutions of different concentrations of  $Ce(NO_3)_3$  were made up in these buffer solutions and put into the collodion bags. The outside solutions

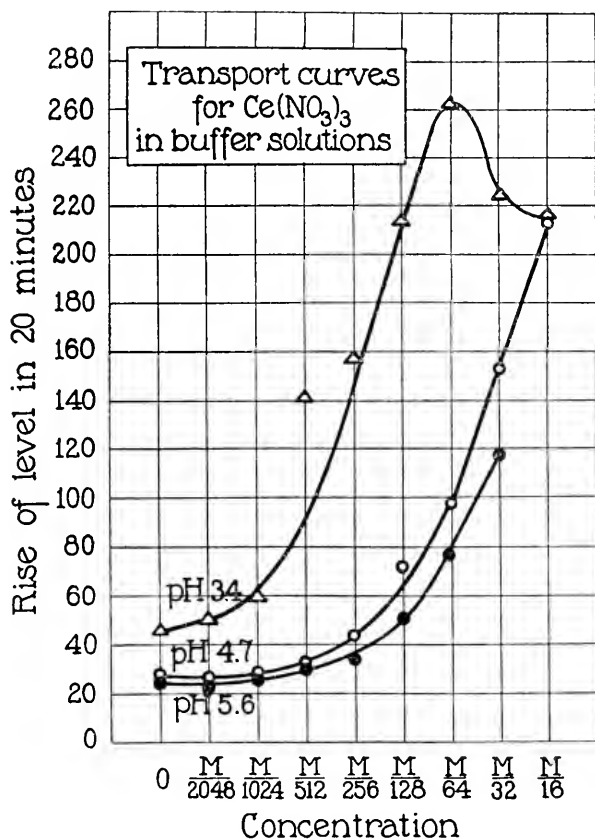


FIG. 6. Influence of  $Ce(NO_3)_3$  on the transport curve in the presence of buffer solutions (mixtures of  $M/50$  acetic acid and  $M/50$  Na acetate).

were the same buffer solutions without the  $Ce(NO_3)_3$ . The curves in Fig. 6 represent the transport values in 20 minutes. We will first direct our attention to the transport curve at pH 4.7. It is clear that at the isoelectric point the transport curve for  $Ce(NO_3)_3$  rises again steeply, thus supporting the idea that the  $Ce(NO_3)_3$  reacts with the

protein in the way suggested, thereby giving rise to a double layer in the pores in which the gelatin is positively and the liquid negatively charged. Table VIII shows that at pH 4.7 there is a high P.D. across the membrane in which the  $\text{Ce}(\text{NO}_3)_3$  solution assumes a positive charge. If now the liquid inside the pores is negatively charged an electrical transport of water from the water side into the solution must occur.

A comparison of the transport curve for  $\text{Ce}(\text{NO}_3)_3$  of pH 4.7 in Fig. 6 with that in Fig. 4 shows that the latter is higher. This finds its explanation in the fact (evident by a comparison of Tables VIII and VII) that the P.D. across the membrane, *i.e.* the value  $E$ , is less in the experiment with buffer salt in Fig. 6 than without it in Fig. 4.

TABLE VIII.

P.D. in millivolts across the collodion-gelatin membrane between solutions of  $\text{Ce}(\text{NO}_3)_3$  made up in buffer solution, and buffer solution free from  $\text{Ce}(\text{NO}_3)_3$ . The  $\text{Ce}(\text{NO}_3)_3$  solution is always positively charged.

Concentration of $\text{Ce}(\text{NO}_3)_3$ .	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16
pH	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
4.7	0	2.0	4.0	7.0	11.5	16.0	21.0	27.0	34.0
5.6	0	1.0	2.5	4.5	7.5	11.5	17.0	23.0	28.0
3.4	2.0	10.5	17.5	26.0	29.0	32.0	35.0	38.0	44.0

The transport curve at pH 3.4 is higher than at pH 4.7 and the transport curve at pH 5.6 is lower than at pH 4.7 (Fig. 6). Table VIII shows that this finds its explanation in the fact that the P.D. across the membrane varies correspondingly with the pH.

As a consequence it seems possible that the  $\text{Ce}(\text{NO}_3)_3$  causes the positive charge of gelatin by forming a dissociable salt with gelatin in which the positive ion is a complex gelatin-Ce ion. In other words, the salt reacts with isoelectric gelatin in a similar way as acid does. If this assumption is correct the P.D. between gelatin and bounding  $\text{Ce}(\text{NO}_3)_3$  solution must be ascribed to a Donnan equilibrium, in which the  $\text{Ce}(\text{NO}_3)_3$  plays a similar rôle as the HCl.

The difference between gelatin chloride and the hypothetical gelatin- $\text{Ce}(\text{NO}_3)_3$  salt is this that it is much easier to remove by washing the  $\text{Ce}(\text{NO}_3)_3$  from powdered gelatin than it is to remove the HCl.

In an analogous way we must assume that isoelectric gelatin can combine loosely with  $\text{Na}_4\text{Fe}(\text{CN})_6$  whereby negatively charged complex gelatin- $\text{Fe}(\text{CN})_6$  ions and positively charged Na ions are formed.

#### SUMMARY AND CONCLUSIONS.

1. It has been shown in previous publications that when solutions of different concentrations of salts are separated by collodion-gelatin membranes from water, electrical forces participate in addition to osmotic forces in the transport of water from the side of the water to that of the solution. When the hydrogen ion concentration of the salt solution and of the water on the other side of the membrane is the same and if both are on the acid side of the isoelectric point of gelatin (*e.g.* pH 3.0), the electrical transport of water increases with the valency of the cation and inversely with the valency of the anion of the salt in solution. Moreover, the electrical transport of water increases at first with increasing concentration of the solution until a maximum is reached at a concentration of about  $M/32$ , when upon further increase of the concentration of the salt solution the transport diminishes until a concentration of about  $M/4$  is reached, when a second rise begins, which is exclusively or pre-eminently the expression of osmotic forces and therefore needs no further discussion.

2. It is shown that the increase in the height of the transport curves with increase in the valency of the cation and inversely with the increase in the valency of the anion is due to the influence of the salt on the P.D. ( $E$ ) across the membrane, the positive charge of the solution increasing in the same way with the valency of the ions mentioned. This effect on the P.D. increases with increasing concentration of the solution and is partly, if not essentially, the result of diffusion potentials.

3. The drop in the transport curves is, however, due to the influence of the salts on the P.D. ( $\epsilon$ ) between the liquid inside the pores of the gelatin membrane and the gelatin walls of the pores. According to the Donnan equilibrium the liquid inside the pores must be negatively charged at pH 3.0 and this charge is diminished the higher the concentration of the salt. Since the electrical transport is in proportion to the product of  $E \times \epsilon$  and since the augmenting action of

the salt on  $E$  begins at lower concentrations than the depressing action on  $\epsilon$ , it follows that the electrical transport of water must at first rise with increasing concentration of the salt and then drop.

4. If the Donnan equilibrium is the sole cause for the P.D. ( $\epsilon$ ) between solid gelatin and watery solution the transport of water through collodion-gelatin membranes from water to salt solution should be determined purely by osmotic forces when water, gelatin, and salt solution have the hydrogen ion concentration of the isoelectric point of gelatin ( $\text{pH} = 4.7$ ). It is shown that this is practically the case when solutions of  $\text{LiCl}$ ,  $\text{NaCl}$ ,  $\text{KCl}$ ,  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ ,  $\text{BaCl}_2$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{MgSO}_4$  are separated by collodion-gelatin membranes from water; that, however, when the salt has a trivalent (or tetravalent?) cation or a tetravalent anion a P.D. between solid isoelectric gelatin and water is produced in which the wall assumes the sign of charge of the polyvalent ion.

5. It is suggested that the salts with trivalent cation, *e.g.*  $\text{Ce}(\text{NO}_3)_3$ , form loose compounds with isoelectric gelatin which dissociate electrolytically into positively charged complex gelatin- $\text{Ce}$  ions and negatively charged  $\text{NO}_3$  ions, and that the salts of  $\text{Na}_4\text{Fe}(\text{CN})_6$  form loose compounds with isoelectric gelatin which dissociate electrolytically into negatively charged complex gelatin- $\text{Fe}(\text{CN})_6$  ions and positively charged  $\text{Na}$  ions. The Donnan equilibrium resulting from this ionization would in that case be the cause of the charge of the membrane.



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DOES THE KINETICS OF TRYPSIN DIGESTION DEPEND  
ON THE FORMATION OF A COMPOUND BETWEEN  
ENZYME AND SUBSTRATE.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 13, 1922.)

Trypsin, in common with many other enzymes, possesses the peculiarity that the rate of formation of the products of hydrolysis does not increase in proportion<sup>1</sup> to the substrate concentration, as is demanded by the law of mass action, but increases more slowly than the substrate concentration and eventually becomes nearly independent of it.<sup>2</sup> It is necessary to assume, therefore, either that the mass law in its simple form does not hold or that the concentration in grams per liter does not represent the "active" concentration of substrate. A very similar phenomenon is quite common in ordinary catalytic reactions. In this case it has usually been assumed that the mass law is valid but that the concentration to be used in the calculation is not the total concentration of the substance taken but that of some particular molecular species which is formed from this substance in solution. In the case of acid hydrolysis, for instance, the action is assumed to be equal to the concentration, not of the acid itself, but of the hydrogen ions. In this case the assumption is capable of verification since the concentration of hydrogen ions may be determined by several independent methods which give approximately the same results, all verifying the assumption. If this were not the case, the kinetics of acid hydrolysis would be more difficult to interpret than the kinetics of enzyme reactions. In the case of enzymes, however, it has usually been assumed, following the

<sup>1</sup> Bayliss, W. M., *Arch. Sc. Biol.*, 1904, xi, suppl., 261.

<sup>2</sup> In the case of alkali or acid hydrolysis this is not true. The rate of digestion in the absence of enzyme is proportional to the gelatin concentration. See Northrop, J. H., *J. Gen. Physiol.*, 1921, iii, 715.

suggestions of Henri<sup>3</sup> and of Brown<sup>4</sup> that the law of mass action in its simple form does not hold but that the velocity of the reaction depends upon the decomposition of a compound between the enzyme and substrate. There is a large amount of evidence that a compound is first formed in many chemical reactions and it has even been stated (Kekule) that no reaction can take place without an addition compound first being formed between the reacting substances. It is quite probable that such a compound is formed in the case of enzyme reactions. The question is whether a sufficient amount of the compound is present at any time to make the kinetics of the reaction depend on the concentration of the compound rather than on the concentration of the reacting substances. Henri,<sup>3</sup> and Michaelis and Menten<sup>5</sup> have attempted to explain the kinetics of invertase hydrolysis by the assumption that the enzyme and substrate combine, according to the law of mass action, to form a compound which subsequently decomposes, liberating the free enzyme and the products of the reaction.<sup>6</sup> It is also assumed that the velocity of hydrolysis depends on the concentration of this compound. It was pointed out by the writer,<sup>7</sup> that if the velocity of hydrolysis depended on the amount of compound formed, then the concentration of substrate required to give the maximum velocity of hydrolysis (*i.e.* to "saturate" the enzyme) should increase with increasing concentrations of enzyme, since it will obviously require more substrate to saturate 100 units of enzyme than it will require to saturate 1 unit. The experiments did not confirm the expectation. It was found that the relative velocity of hydrolysis of different substrate concentrations is always the same, within the experimental error, no matter what enzyme concentration is used (provided the same amount is used with each substrate concentration). It was stated in the article referred to that this was contradictory to the assumption that there was a com-

<sup>3</sup> Henri, V., *Compt. rend. Acad.*, 1902, xxxcv, 916; *Z. physik. Chem.*, 1905, li, 19.

<sup>4</sup> Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

<sup>5</sup> Michaelis, L., and Menten, M., *Biochem. Z.*, 1913, xlix, 333.

<sup>6</sup> It has been shown by Simons in Nelson's laboratory that the method used by Michaelis to measure the initial velocity gives values which cannot be used over the entire course of the reaction.

<sup>7</sup> Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 595.

pound formed between the enzyme and substrate, since if this were true a greater amount of substrate would be required to saturate a greater amount of enzyme. The experiment, however, is not conclusive, since if it is assumed, as was done by Michaelis and Menten, that the amount of substrate combined with the enzyme is negligibly small, then the difference in concentration of substrate necessary to saturate different amounts of enzyme would be entirely too small to detect experimentally. As far as the relation between the rate of hydrolysis and the concentration of enzyme or substrate is concerned, therefore, the facts may be accounted for by the assumption of an intermediate compound.

It has been shown in a preceding paper<sup>8</sup> that the inhibiting action of the products of the reaction on the trypsin is in quantitative agreement with the assumption that the enzyme and the inhibiting substance combine to form a compound which is inactive and that the rate of hydrolysis is proportional to the concentration of *uncombined* trypsin. It has also been shown that the same assumption will account quantitatively for the protective action of the inhibiting substances when the spontaneous inactivation of the enzyme is followed. The fact that the inhibiting substance protects the enzyme from decomposition is strong evidence that the inhibiting substance combines with the enzyme. In the presence of the substrate, however, the enzyme becomes inactivated at the same rate as the "pure" enzyme<sup>8</sup> (see Ringer).<sup>9</sup> These facts render it unlikely that the enzyme is combined with the substrate. The present paper contains the results of experiments planned to determine whether or not the action of the enzyme with different concentrations of substrate and of inhibiting substances can be accounted for on the assumption of a compound between the enzyme and substrate. The observed facts cannot be accounted for on the basis of the formation of a compound between enzyme and substrate, if it be assumed that this compound is governed by the law of mass action.

<sup>8</sup> Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 266.

<sup>9</sup> Ringer, W. E., *Z. physiol. Chem.*, 1921, cvi, 107.

*Experimental Methods.*

The rate of hydrolysis was followed by means of the change in conductivity of the solution as already described.<sup>8</sup> The experiments were all conducted at a pH of 6.0.

*Trypsin.*—The trypsin was a sample of Fairchild's trypsin and was purified for use by dialysis under pressure.

Cooper's gelatin was used and was rendered ash-free by washing at the isoelectric point as described by Loeb.<sup>10</sup> The inhibiting solution was made by allowing trypsin to completely digest gelatin and then concentrating the solution *in vacuo*.

*Method of Measuring the Rate of Hydrolysis.*—In order to obtain a correct measure of the rate of hydrolysis it is necessary to compare the reactions at the same stage. The rate of digestion decreases rapidly with the progress of digestion for two reasons: first, the concentration of substrate is decreasing; second, the concentration of active enzyme is decreasing owing to the inhibiting action of the products of digestion. If the reactions are compared at a point of equal percentage hydrolysis, the change in substrate concentration is corrected for but the change in enzyme concentration will be very different. The small amount of enzyme will be inhibited to a larger extent than the large amount. If the reactions are compared after equal times, both conditions are varied. If, however, the time to cause a very small amount of hydrolysis is taken, the change in substrate concentration may be considered negligible and the effect on the enzyme will be small and nearly the same in both cases. This method, therefore, gives the most significant value.

The result of an experiment with 1 and 5 per cent gelatin and 1 and 10 units of trypsin is shown in Figs. 1, 2, and 3, in which the increase in specific conductivity of the solution has been plotted against the time in hours. Table I gives the time required to cause an equal percentage of the total change in the two gelatin concentrations with the different enzyme concentrations. The table shows that the time required for the hydrolysis to be completed to any given percentage in the two solutions, is not the same (as would be predicted by the monomolecular formula), but is very much greater for the 5 than for

<sup>10</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 237.



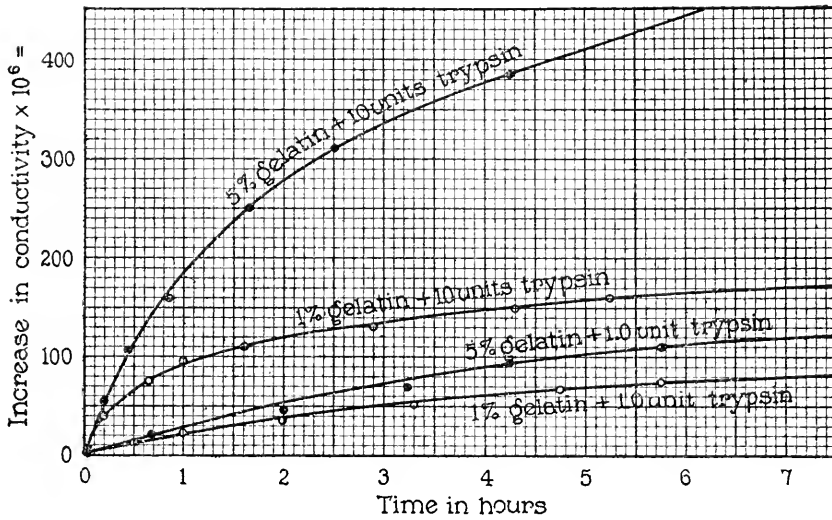


FIG. 1. Rate of digestion of 5 per cent and 1 per cent gelatin with 1 unit and 10 units trypsin as followed by the increase in conductivity.

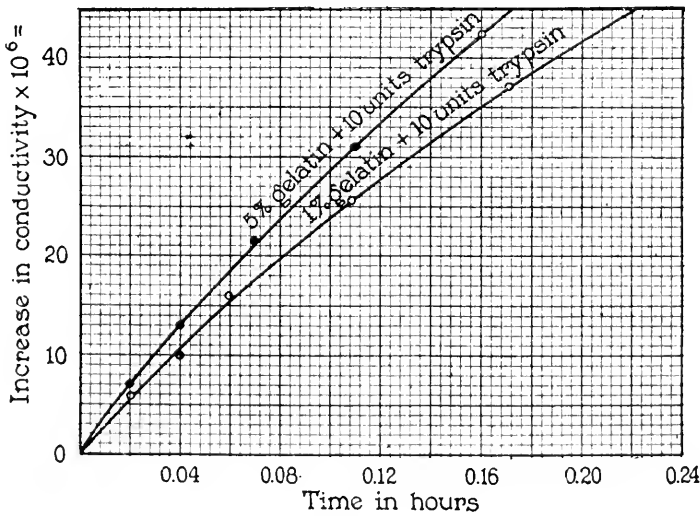


FIG. 2. Large scale of the beginning of Fig. 1. 10 units trypsin.

the 1 per cent gelatin. The difference is greater with the small amount of trypsin than it is with the larger. The same result is shown in Table II in which the change in conductivity after an equal time is given. Here the ratio of the change in the 1 per cent gelatin compared to the change in the 5 per cent gelatin is much smaller when 10 units of trypsin are used than when 1 unit is used. The results when calculated in this way then seem to show that 1 unit of trypsin becomes "saturated" with gelatin at a lower concentration

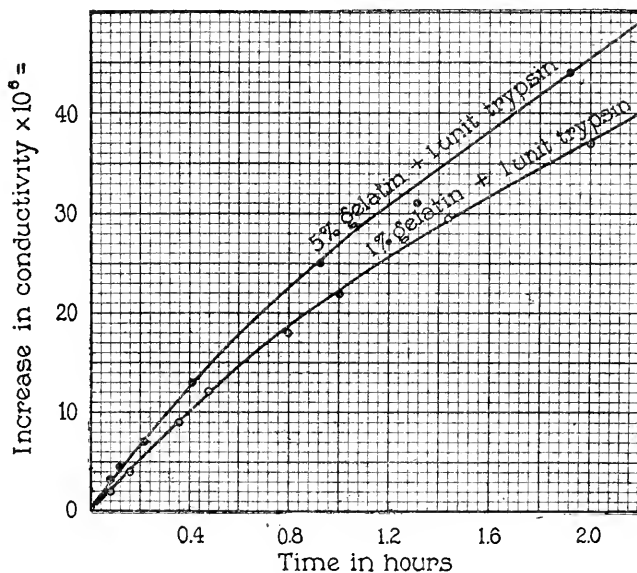


FIG. 3. Large scale of the beginning of Fig. 1. 1 unit trypsin.

of gelatin than do 10 units. This result, however, is not due to the "saturation" or combination of the enzyme with the gelatin but to the fact that different stages of the reaction are being compared. That this is actually the case is shown in Table III, in which the time required to cause the same amount of hydrolysis is given. In this case the amount of products formed is the same in both solutions (*i.e.* the stage of the reaction compared is the same) and, as the table shows, the relative velocity of hydrolysis of the 5 per cent gelatin compared to the 1 per cent gelatin is independent of the amount of

trypsin used. The table also shows that this ratio is constant if the first part of the curve is used but later decreases (*i.e.*, the 5 per cent gelatin is hydrolyzed relatively more and more rapidly), as would be expected, since after any appreciable amount of the gelatin is hydro-

TABLE I.

*Time Required for Equal Percentage Hydrolysis of 1 and 5 Per Cent Gelatin Solutions When Compared with (a) 1 Unit of Trypsin, and (b) 10 Units of Trypsin.*

Complete hydrolysis of 1 per cent gelatin = increase in conductivity of  $0.5 \times 10^{-3}$  reciprocal ohms.

Hydrolysis.	Actual change in conductivity.	1 per cent gelatin hydrolyzed with		Actual change in conductivity.	5 per cent gelatin hydrolyzed with		Relative rate of hydrolysis of 1 per cent gelatin compared to 5 per cent gelatin.	
		1 unit trypsin. (a)	10 units trypsin. (b)		1 unit trypsin. (c)	10 units trypsin. (d)	1 unit trypsin. $\frac{c}{a}$	10 units trypsin. $\frac{d}{b}$
<i>per cent</i>	<i>reciprocal ohms</i> $\times 10^6$	<i>hrs.</i>	<i>hrs.</i>	<i>reciprocal ohms</i> $\times 10^6$	<i>hrs.</i>	<i>hrs.</i>		
1	5	0.20	0.018	25	0.90	0.085	4.5	4.7
2	10	0.40	0.037	50	2.24	0.17	5.6	4.6
5	25	1.16	0.104	125	7.50	0.57	6.5	5.5

TABLE II.

*Change in Conductivity after Equal Time Intervals.*

Time elapsed.	1 per cent gelatin +		5 per cent gelatin +		Ratio, change in 1 per cent gelatin change in 5 per cent gelatin tested with	
	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.
<i>hrs.</i>	<i>reciprocal ohms</i> $\times 10^6$	<i>reciprocal ohms</i> $\times 10^6$	<i>reciprocal ohms</i> $\times 10^6$	<i>reciprocal ohms</i> $\times 10^6$		
0.10	2.5	24	3.5	29	0.7	0.8
0.50	12.5	70	15	115	0.8	0.6
1.00	22	95	28	180	0.8	0.5
3.00	52	135	67	335	0.8	0.4

lyzed the two concentrations are no longer as 5:1 but as  $5-a:1-a$ . As soon as  $a$  becomes appreciably large compared to 1, the ratio will evidently increase, as  $a$  increases. The ratio of the time required to cause a given change will therefore decrease. This method may,

therefore, be used to determine the relative rate of digestion of different gelatin concentrations provided the change used as end-point is so small that the gelatin concentration can be assumed to remain constant during the course of the experiment.

TABLE III.

*Time Required to Cause an Equal Change in the Conductivity of 1 and 5 Per Cent Gelatin Solutions with 1 Unit of Trypsin and 10 Units of Trypsin.*

Increase in conductivity.	1 per cent gelatin +		5 per cent gelatin +		Ratio, time for change in 5 per cent gelatin time for equal change in 1 per cent gelatin with	
	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.
<i>reciprocal ohms <math>\times 10^6</math></i>	<i>hrs.</i>	<i>hrs.</i>	<i>hrs.</i>	<i>hrs.</i>		
5	0.19	0.018	0.15	0.014	0.79	0.78
20	0.87	0.08	0.69	0.066	0.79	0.805
50	2.85	0.27	2.05	0.19	0.72	0.71
75	5.75	0.60	3.50	0.30	0.61	0.50

*Influence of the Viscosity of the Solution.*

It has been suggested that the anomalous results obtained by increasing the concentration of substrate are due to the increased viscosity of the solution. That this is not the cause of the retardation in the present experiments is shown in Table IV which gives the results of an experiment performed with the same gelatin solution which had been kept at 25°C. for varying lengths of time. The viscosity increases slowly under these conditions. As the table shows, the gelatin digests at the same rate whether it has a viscosity of 2.5 times that of water or of 11 times that of water. The physical properties of the gelatin solution evidently have little or no effect on the rate at which it digests.

*Influence of the Substrate Concentration.*

The results of a series of experiments with varying enzyme and gelatin concentrations are given in Table V. The hydrolysis was followed by the change in conductivity and velocity is taken as the reciprocal of the time required to cause an increase of conductivity

TABLE IV.

*Viscosity and Rate of Digestion.*

2 per cent gelatin, pH 6.0, specific conductivity  $1 \times 10^{-3}$  (adjusted with NaCl) was heated to 50°C. and cooled rapidly to 25°C. Viscosity was determined at intervals at 25°C. and rate of digestion determined by adding 1 cc. of trypsin to 25 cc. gelatin and following change in conductivity. Increase in formol titration after 1 hr. was also determined.

Viscosity (H <sub>2</sub> O = 1.0),		Time to change 10 points.	Formol per 5 cc. after 1 hr. (N/50 NaOH).
At beginning.	After digestion.		
		<i>hrs. × 10<sup>2</sup></i>	<i>cc.</i>
2.45			
2.90	1.4	70	2.90
		68	2.90
3.8	1.55	70	2.90
3.9	1.5	69	2.95
4.6	1.6	64	2.95
		76	2.97
7.3	1.7	80	2.95
		80(?)	2.90
		62	
11.3	1.8	70	2.95

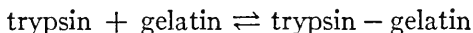
TABLE V.

*Comparison of Observed and Calculated Results with Varying Gelatin and Trypsin Concentrations.*

Concentration of gelatin, S.	Rate of hydrolysis $\left( = \frac{100}{T \text{ hrs. to change 5 points in conductivity}} \right) = C$ with enzyme concentrations (E).					
	E = 30.0		E = 5.0.		E = 3.0.	
	Observed.	Calculated.*	Observed.	Calculated.*	Observed.	Calculated.*
<i>per cent</i>						
6.0	27.3	27.7	4.9	4.6	2.7	2.7
3.0	28.4	25.7	4.6	4.3	2.4	2.6
1.5	25.6	22.5	4.0	3.7	2.2	2.2
0.75	17.5	18.0	3.3	3.0	1.8	1.8
0.38	13.1	12.0	2.4	2.0	1.3	1.3

\* Calculated from formula  $C = \frac{E S}{K' + S}$ ,  $K' = 0.5$ . The values for E are given at the head of the table.

equivalent to 5 points on the bridge. The table shows that the velocity of hydrolysis increases much more slowly than the substrate concentration and becomes practically independent of it in concentrations of more than 3 per cent.<sup>1</sup> The calculated figures were obtained by assuming that the trypsin and gelatin combined according to the reaction



and that the rate of hydrolysis was proportional to the concentration of the *trypsin-gelatin compound*.

Applying the law of mass action to this equilibrium we would have

$$\frac{(E - C) \cdot a S}{C} = K V$$

or

$$C = \frac{E S}{K' + S}$$

in which  $E$  equals total amount of trypsin;  $C$ , combined trypsin (= combined gelatin);  $S$ , amount of gelatin;  $a$ , a proportionality factor to change the units of concentration to those of rate of hydrolysis;  $K$ , the equilibrium constant;  $V$ , the volume of solution; and  $K'$ , a new constant equal to

$$\frac{K V}{a}$$

It will be seen that if  $C$  is considered negligibly small, compared to  $E$ , as well as to  $S$ , the equation reduces to  $\text{Rate} = kC = KES$  which is the ordinary form of the law of mass action.

#### *Evaluation of the Constants.*

Since it is assumed that the rate of hydrolysis is proportional to the amount of the gelatin-trypsin compound ( $C$ ) present,  $C$  is the observed velocity.

$E$ , the total amount of trypsin, cannot be determined directly but is taken as a value slightly larger than the maximum value obtained for  $C$ , when the substrate concentration is such that the hydrolysis proceeds at the maximum rate. According to the hypothesis, this maximum value is due to the fact that practically all the enzyme is combined, and since  $C$  (the rate of hydrolysis) is a measure of the amount combined,  $E$  must be very slightly larger.  $E$  is, therefore, an arbitrary constant. After a value for  $E$  has been determined for a given amount of trypsin solution, the value to be used with any other amount of trypsin will be propor-

tional to the relative amount of trypsin taken, *i.e.*, if  $E_0$  is the value used when 1 cc. of trypsin is taken then  $nE_0$  will be the value for  $n$  cc.

$K'$  is determined by substituting the values of  $C$ ,  $E$ , and  $S$  and solving for  $K'$ . It is, therefore, a second arbitrary constant.

Table V shows that the calculated and observed values are concordant. It also shows that the relative rate of hydrolysis of any two gelatin concentrations with any one trypsin concentration is independent of the value of this trypsin concentration.

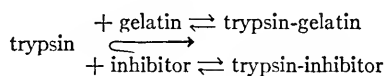
The results when the enzyme or substrate concentrations are varied, therefore, agree with those predicted by the assumption that the enzyme and gelatin combine to form a compound and that the rate of hydrolysis is proportional to the concentration of this compound.

The equation which was used to calculate these values, however, contains two arbitrary constants and is of a form to fit any curve which at first shows direct proportionality and then approaches a maximum value. It is not surprising, therefore, that the calculated and observed results agree. The test of an equation of this type is to see whether or not it will fit the results of an experiment other than the one from which the values of the constants was originally obtained. As will be seen below, this is not the case; the equation breaks down when the experiment is performed in the presence of inhibiting substances.

#### *Influence of the Gelatin Concentration on the Retarding Effect of Inhibiting Substances.*

It was found<sup>8</sup> in studying the influence of the inhibiting substance on the rate of digestion that the experiments agreed with the assumption that the enzyme and inhibitor combined to form a compound that was inactive and that the rate of hydrolysis was proportional to the concentration of *free enzyme*. It was also found that there was direct evidence that the inhibitor affects the enzyme and not the substrate. The experiment summarized in Table V, however, if taken alone, shows that the influence of the substrate concentration agrees with the assumption that the rate of hydrolysis is proportional to the amount of *enzyme combined* with the substrate. It is evident that both assumptions cannot be correct.

The direct experimental evidence shows conclusively that the enzyme and the inhibiting substances are combined to form a highly dissociated compound, even though the agreement of the experiments with the results calculated from the law of mass action be considered to be accidental. If the substrate is also combined with the enzyme it should be possible, by increasing the substrate concentration sufficiently, to cause all the enzyme to combine with the substrate. In other words, the higher the substrate concentration the less should be the effect of the inhibiting substance. This may be seen from the following equation:



Increasing the concentration of gelatin will cause the equilibrium to be shifted in the direction of the large arrow; *i.e.*; it will cause the amount of trypsin combined with the inhibitor to become less. This will be true even though the equilibrium is not one which follows the law of mass action, as long as the equilibrium is reversible and the trypsin-inhibitor compound widely dissociated. It has already been found that the trypsin-inhibitor compound is readily dissociated irrespective of any assumption as to the nature of the compound. Table VI is a summary of experiments made with constant amounts of trypsin and inhibiting substance and increasing concentrations of gelatin. The figures are the averages of four to six determinations. The table shows that the retardation due to the inhibiting substance is independent of the gelatin concentration. In order to account for this result, if the velocity of hydrolysis depends on a trypsin-gelatin compound, it is necessary to assume that the trypsin-inhibitor compound is only very slightly dissociated, and that the inhibiting substance and trypsin are present in about the same concentration. The figures under Calculated I were obtained by means of the law of mass action based on these assumptions. They approximate the experimental values and agree with the experimental result that the percentage retardation is independent of the gelatin concentration. If it be assumed, as was done by Michaelis and Menten, that the inhibiting substance is present in very much higher concentration than the enzyme, the figures given under Calculated II are obtained. They are evidently incompatible with the experiment.



TABLE VI.

*Influence of the Gelatin Concentration on the Retardation Caused by the Presence of a Constant Amount of Inhibiting Substances.*

Concentration of gelatin, S.	C = rate of hydrolysis in					Ratio of rate of hydrolysis of control inhibitor Observed.
	Control solution.		Solution containing inhibitor.			
	Observed.	Calculated.*	Observed.	Calculated (I).*	Calculated (II).†	
<i>per cent</i>						
8	10.0	9.5	7.1	6.6	[7.1]	1.43
4	10.0	9.0	7.0	6.3	5.4	1.43
2	9.6	8.1	6.8	5.7	3.7	1.41
1	7.3	6.8	5.0	4.7	2.3	1.46

\* Calculated by equation  $C = \frac{S(E-I)}{K+S}$ .  $E = 10.1$ ,  $I = 3.0$  (units inhibitor),  $K = 0.5$  (derived by assuming that inhibitor-enzyme compound is very little dissociated).

† Calculated by equation  $C = \frac{ES}{K''+S} = 10.1$ ,  $K'' = 3.4$ , derived by assuming that the amount of inhibitor combined with the enzyme is negligible compared to total amount of inhibitor, and that inhibitor enzyme compound is widely dissociated.

This equation is derived as follows: Let  $S$  = total substrate concentration,  $E$  total enzyme concentration,  $C$  enzyme combined with substrate,  $J$  enzyme combined with inhibitor, and  $I$  total inhibitor.

Then if the amount of substrate combined with the enzyme is negligibly small compared to the total amount of substrate and if the same is true of the inhibitor, the mass action expressions for the two equilibria are

$$S(E - C - J) = KC \quad (1) \quad I(E - C - J) = kJ \quad (2)$$

$$J = \frac{ES - KC - SC}{S}$$

Substituting this value of  $J$  in (2) and simplifying  $C = \frac{ES}{\frac{IK}{k} + K + S}$  in which

$K$  = equilibrium constant of substrate-enzyme equilibrium, and  $k$  = equilibrium constant for inhibitor-enzyme compound. Since in this experiment  $I$ ,  $K$ , and  $k$  are all constant they may be combined to a new constant  $K''$  and  $C = \frac{ES}{K''+S}$ .

*Effect of Varying the Amount of Trypsin or Inhibiting Substance.*

It has been shown above that in order to account for the fact that the percentage retardation is independent of the substrate concentration, it is necessary to assume that the inhibitor-trypsin compound is only slightly dissociated. This assumption, as has already been pointed out, is contradicted by the experiments in which the amount of trypsin or inhibitor is varied. This is shown in Tables VII and VIII. In these tables the results under Calculated I were obtained from the equation used to calculate the results in Table VI, and which is derived by means of the assumption that the inhibitor-trypsin compound is only slightly dissociated. The table shows that the formula will not serve even as a first approximation in spite of the fact that it contains three arbitrary constants. The figures given under Calculated II were obtained by aid of the assumption that the enzyme-inhibitor compound is widely dissociated and that the rate of hydrolysis is proportional to the free enzyme.<sup>11</sup> They agree well with the experimental values.

These experiments show that the results obtained when the gelatin, inhibitor, and trypsin concentrations are all varied cannot be accounted for on the assumption that the trypsin becomes saturated with substrate. They seem to be conclusive even though it is assumed that the equilibria are not governed by the law of mass action, since in order to explain one set of experiments (gelatin constant, trypsin or inhibitor varied) it is necessary to suppose that the trypsin-inhibitor compound is widely dissociated while in the other set of experiments (trypsin and inhibitor constant, gelatin varied) it is necessary to suppose that the same compound is very slightly dissociated. This is true irrespective of the quantitative law that is assumed to govern the equilibrium. There is much more direct experimental evidence in favor of the trypsin-inhibitor compound than of the trypsin-gelatin compound.

There is no doubt on the other hand that the rate of hydrolysis does not increase in proportion to the gelatin concentration as expressed in grams per liter. If it is assumed then that the reaction is

<sup>11</sup> For the derivation of this equation see Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 230.

TABLE VII.

*Effect of Increasing the Concentration of Inhibitor. 2 Per Cent Gelatin.*

Inhibiting solution.	Rate of hydrolysis = C.		
	Observed.	Calculated (I).*	Calculated (II).†
cc.			
0.0	2.3	2.3	[2.3]
0.125	1.9	[1.9]	1.81
0.25	1.56	1.5	1.45
0.50	1.10	0.72	1.00
1.0	0.65	<0.10	0.58
2.0	0.33	<0.01	0.32

\* Calculated from  $C = \frac{S(E - I \text{ cc.})}{K + S}$ ; i.e., enzyme-inhibitor compound very slightly dissociated; velocity proportional to combined enzyme (C).  $E = 2.9$ ,  $S = 2$ ,  $K = 0.5$ ,  $I = 4.0$  units inhibitor per cc. of solution.

$$\dagger \text{ Calculated from } Q = \sqrt{\left(\frac{d + K - E}{2}\right)^2 + K E} - \frac{d + K - E}{2}$$

Assumptions: (1) enzyme-inhibitor compound widely dissociated. (2) Rate of hydrolysis proportional to free enzyme (Q).  $K = 2.8$ ,  $E = 2.3$ ,  $d = \text{cc. inhibiting solution} \times 10$ .

TABLE VIII.

*Effect of Varying Enzyme Concentration with Constant Gelatin and Constant Inhibitor Concentrations.*

Gelatin 2 per cent. 25 cc. + 1 cc. inhibiting solution + noted cc. trypsin solution.

Trypsin.	$E_1$	$E_2$	Rate of hydrolysis.		
			Observed.	Calculated.*	Calculated.†
cc.					
1.1	13.0	11.0	7.4	[7.4]	[7.4]
0.55	6.6	5.5	3.2	2.3	3.0
0.30	3.6	3.0	1.4	<0.01	1.35
0.15	1.8	1.5	0.80	<0.01	0.70

\* Calculated by formula  $C = \frac{S(E_1 - I)}{K + S}$ ,  $S = 2$ ,  $I = 3.7$ ,  $K = 0.5$ ,  $E_1$  as in table. Assumptions: (1) enzyme-inhibitor compound slightly dissociated; (2) velocity proportional to combined enzyme.

† Calculated by same formula as (II), Table VII.  $d = 5.0$ .  $E_2$  as in table above.

governed by the law of mass action and that the velocity of hydrolysis is really proportional to the concentration of free trypsin and protein, it is necessary to suppose that the reaction is confined to some particular molecular species present in the protein solution or to introduce a "catalysis" coefficient to express the ratio of actual concentration to "active" concentration as has been done in the case of hydrogen ion by Schreiner.<sup>12</sup> The work of Loeb, Michaelis, Sørensen, Robertson, and others has shown that proteins in solution are ionized so that it would be natural to suppose that the speed of reaction is proportional to the concentration of protein ions instead of to the total concentration of protein. It has been found that in the case of pepsin hydrolysis this accounted for the difficulty both as regards differences in the concentration of protein and the effect of the hydrogen ion concentration of the solution. In the present case, however, the ionic concentration, as measured by the conductivity of the solution, increases more rapidly than the rate of hydrolysis but less rapidly than the concentration so that the anomaly is only partly corrected for.

It has been found by von Euler and Svanberg<sup>13</sup> in the case of invertase that the retardation due to inhibiting substances is independent of the substrate (sugar) concentration so that in the case of this enzyme also the evidence is contradictory to the assumption of a substrate-enzyme compound.

#### *Hydrolysis of Mixtures of Casein and Gelatin.*

The rate of hydrolysis of casein solutions increases less rapidly than the concentration of casein, just as in the case of gelatin. This is shown in Table IX. It is evident that increasing the concentration of casein above 4 per cent has little or no effect on the rate of digestion. According to the saturation hypothesis the trypsin must, therefore, be "saturated" with casein when the latter is at a concentration of 4 per cent or more. It is interesting to consider the digestion of a mixture of casein and gelatin from the points of view

<sup>12</sup> Schreiner, E., *Z. anorg. Chem.*, 1921, cxvi, 102.

<sup>13</sup> von Euler, H., and Svanberg, O., *Fermentforschung*, 1921, iv, 142.

of the various possible assumptions.<sup>14</sup> The following possibilities present themselves and may be compared with the experiment shown in Fig. 4 and Table X. The values are the mean of 4 to 6 determinations.

TABLE IX.

*Effect of Increasing Casein Concentrations on the Amount of Casein Digested.*  
pH 8.0. Phosphate buffer. 34°C.

Casein concentration, <i>per cent</i> .....	0.5	1.0	2.0	3.0	5.0
Increase of amino nitrogen per cc. solution after 1 hr., <i>cc</i> .....	0.20	0.33	0.45	0.54	0.55

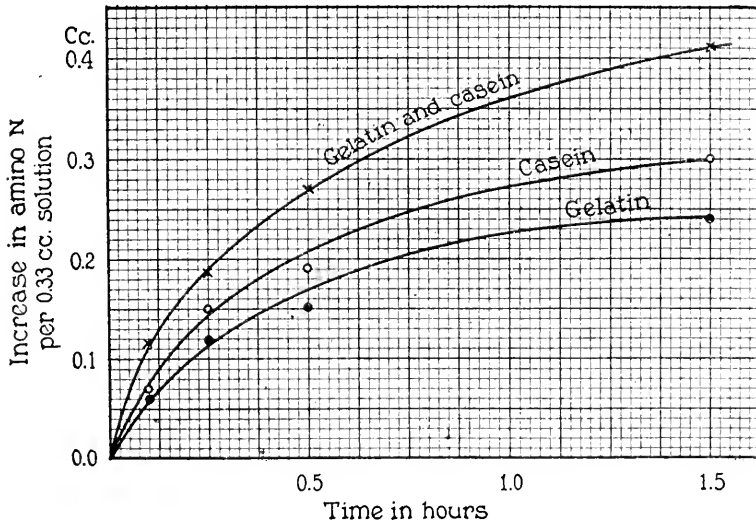


FIG. 4. Digestion curves for 4 per cent casein, 3 per cent gelatin, and a mixture containing both 3 per cent gelatin and 4 per cent casein with the same concentration of trypsin.

<sup>14</sup> This case was considered by Henri and des Bancels (Henri, V., and des Bancels L., *Compt. rend. Soc. biol.*, 1903, lv, 866), who, however, failed to distinguish between the *rate of hydrolysis* and the *amount of hydrolysis*.

TABLE X.

*Rate of Hydrolysis of Casein, Gelatin, and a Mixture of Casein and Gelatin.*

4 cc. dialyzed trypsin were added to each solution at 34°C. 5 cc. samples were removed after 0.10, 0.25, 0.50, 1.50, and 3.0 hrs. and run into 25 cc. of water containing 10 cc. 0.20 N HCl. 2 cc. of this solution (equivalent to 0.33 cc. of original solution) were analyzed for amino nitrogen by Van Slyke method.

Increase in $\text{NH}_2 \text{N}$ . (a)	Time required to cause an increase of amino nitrogen noted under a in						
	Casein solution.	Gelatin solution.	Casein + Gelatin.				
cc.	hrs.	hrs.	hrs.				
0.1	0.15	0.20	0.09				
0.15	0.27	0.40	0.16				
0.20	0.48	0.72	0.28				
Stage of reaction compared.	$\text{Rate of hydrolysis} = \frac{1}{T \text{ hrs.}}$ in						
	Casein solution. (b)	Gelatin solution. (c)	Casein + gelatin.				
			In mixture.	Separately (b + c).			
			0-0.10	6.7	5.0	11.1	11.7
			0-0.15	3.7	2.5	6.2	6.2
0-0.20	2.1	1.4	3.6	3.5			

Casein solution. 4 gm. casein in 100 cc. phosphate buffer.  $\text{m}/10$ , titrated to pH 7.5.

Gelatin solution. 3.5 gm. gelatin in 100 cc. phosphate buffer. pH 7.5.

Gelatin-casein solution. 4 gm. casein + 3.5 gm. gelatin in 100 cc. phosphate buffer as above. pH 7.5.

*I. Rate of Hydrolysis Depends on the Concentration of the Enzyme Substrate Compound.*

(a) The same enzyme acts on both casein and gelatin.

In this case the rate of hydrolysis of the mixture must be less than the rate of hydrolysis of the casein alone, since it has already been assumed, in order to account for the rate-concentration curve, that the enzyme is saturated by 4 per cent casein. The addition of gelatin to the system will therefore remove some enzyme from the casein to combine with the gelatin and since the gelatin hydrolyzes more slowly than the casein, the result will be a decrease in the rate of digestion. This is contradicted by the experiment.

(b) The casein and gelatin are hydrolyzed by different enzymes acting entirely independently.

The amount digested at any time in the mixture must then be equal to the sum of the amounts digested at the same time when the casein and gelatin are hydrolyzed separately. The same result would be predicted if the rate of hydrolysis depended on the concentration of free enzymes. This is also contradicted by the experiment.

(c) The casein and gelatin are acted on by two different enzymes, but the products formed by either enzyme inhibit the action of the other.

The rate of hydrolysis of the mixture, according to this mechanism, will equal the sum of the rates of hydrolysis of the two separate solutions but the amount of hydrolysis at any given time will be slightly less in the mixture than the sum of the two separate solutions. The same result would be predicted if the rate of hydrolysis were proportional to the concentration of free enzymes.

This is the experimental result.

## *II. Rate of Hydrolysis is Proportional to the Concentration of Free Enzyme.*

(a) The same enzyme acts on both the casein and gelatin. This assumption predicts that the rate of hydrolysis of the mixture will be equal to the sum of the rates of the two solutions but the amount of hydrolysis of the mixture will be less than the sum of the two separate solutions. This is the experimental result.

We are, therefore, bound to conclude either, first, that the rate of hydrolysis is proportional to the concentration of free enzyme (*i.e.* that the amount combined is negligibly small), or second, that there are two enzymes at work, each of which is inhibited by the products of hydrolysis formed by the other. This latter assumption is gratuitous unless some independent evidence can be found for the existence of two such enzymes. Many experiments were made from this point of view but no evidence could be found for the existence of two enzymes. The ratio of the rate of hydrolysis of gelatin and casein was always the same within the experimental error of about 1 per cent, no matter how the trypsin preparation was treated.

*Rate of Hydrolysis as Measured Directly by the Disappearance of the Substrate.*

It has been shown above that the rate of formation of the products of hydrolysis of gelatin or casein by trypsin does not increase in proportion to the concentration of substrate but increases much more slowly and becomes independent of the substrate concentration when the latter is more than 2 or 3 per cent. It was also shown that this peculiarity could not be accounted for by assuming the existence of an intermediate compound between the enzyme and substrate nor by the assumption that the hydrolysis was proportional to the ionized protein. In these experiments as in most experiments with enzymes the hydrolysis was followed by determining the amount of the products formed and assuming that the amount of substrate remaining is the difference between the amount of products found at any time and the total amount that can be formed under the most favorable conditions. It is well known that trypsin digestion consists of a series of consecutive reactions since a number of products may be isolated from a digestion mixture which can still be acted on by the enzyme. It seemed possible therefore that the peculiar results discussed above were due to the fact that the increase in the products of reaction does not correctly represent the decrease in the substrate concentration. It is the change in concentration of the latter value that is predicted by the law of mass action. An experiment was therefore performed in which the digestion was followed by determining the increase in amino nitrogen and also the *decrease* in unchanged casein. The results are given in tables XI and XII. The tables show that the two methods give entirely different results. As measured by the increase in amino nitrogen the rate of hydrolysis is practically independent of the casein concentration, whereas when the change in the casein concentration is measured directly the rate of digestion is very nearly proportional to the concentration of casein as demanded by the law of mass action. The constant calculated from the monomolecular formula still shows a drop with increasing hydrolysis. This is more marked in the concentrated than in the dilute solution and is the result expected owing to the inhibiting action of the products of hydrolysis. When the rate of hydrolysis is de-



TABLE XI.

*Effect of Increasing Concentration of Casein on the Rate of Hydrolysis as Measured by the Increase in Amino Nitrogen.*

Casein dissolved in a mixture of M/20  $\text{Na}_2\text{HPO}_4$ ,  $\text{H}_3\text{BO}_3$ , and  $\text{Na}_3$  citrate of pH 8.0. 100 cc. of this solution + 1 cc. dialyzed trypsin at  $34^\circ\text{C}$ . for time noted. Amino nitrogen determined by Van Slyke method on 0.6 cc. of solution as noted.

Increase in amino nitrogen per 0.6 cc. solution containing noted concentration of casein.			
After hrs. at $30^\circ\text{C}$ .	Gm. of casein per 100 cc.		
	2 gm.	4 gm.	6 gm.
hrs.	cc.	cc.	cc.
0.5	0.20	0.20	0.24
1.0	0.25	0.26	0.25
2.0	0.30	0.38	0.42

The figures are the mean of 4 to 6 determinations. The average deviation of the mean is about 5 per cent.

TABLE XII.

*Effect of Increasing Concentration of Casein on Rate of Digestion as Measured by the Decrease in Undigested Casein.*

Casein dissolved in M/20 phosphate, borate, citrate buffer pH 8.0. 100 cc. solution + 1 cc. dialyzed trypsin placed at  $34^\circ\text{C}$ . and 10 cc. samples removed as noted, titrated to pH 4.6, and added to 100 cc. 0.1 N acetate buffer pH 4.6. Precipitate filtered, dried at  $100^\circ\text{C}$ ., and weighed.

		Hrs. at $34^\circ\text{C}$ .			
		0	0.25 hr.	0.50 hr.	1 hr.
Casein per 10 cc. solution, gm.	{ a. ....	0.15	0.08	0.05	0.02
	{ b. ....	0.31	0.19	0.12	0.08
	{ c. ....	0.50	0.31	0.26	0.23
Monomolecular constant for solution.	{ a. ....		1.00	0.96	0.87
	{ b. ....		0.90	0.84	0.60
	{ c. ....		0.85	0.60	0.34
Time to dissolve 0.02 gm. in solution, hrs.	{ a. ....	0.08			
	{ b. ....	0.045			
	{ c. ....	0.025			
$K$ = concentration casein $\times$ time to dissolve 0.02 gm.	{ a. ....	0.0120			
	{ b. ....	0.0140			
	{ c. ....	0.0125			

terminated at the same stage of the reaction by interpolation from the time curves, it is found that the rate of hydrolysis increases in direct proportion to the casein concentration. This is shown by the last line of Table XII. The same result is shown by comparing the monomolecular constants at corresponding values of  $x$ . This experiment shows that when the substrate concentration is measured directly the reaction proceeds according to the law of mass action both as regards the concentration of enzyme and of substrate and that the only divergence from the simple monomolecular formula is due to the fact that the enzyme concentration also changes during the course of the reaction, owing to the effect of the products of the reaction, an effect which can easily be demonstrated directly. The experiment described was repeated several times with the same result. It was also found that if the rate of hydrolysis of gelatin was followed by means of the change in viscosity (which is roughly proportional to the gelatin concentration), the same result was obtained. This indicates that in the case of gelatin as well, the apparent discrepancy from the law of mass action is due to the fact that the reaction is really a series of consecutive reactions and that the change in concentration of the original substance cannot be determined from the increase in the total products of reaction. It will be seen that this mechanism will account also for the fact that the increase in the products of reaction as plotted against the time is occasionally a straight line. Assume, for instance, that the reaction may be written  $A \rightarrow B \rightarrow C$  and that  $C$  is what is determined experimentally. The rate of formation of  $C$  will evidently be proportional to the concentration of  $B$  at any instant, and this in turn will depend on the relation between its rate of formation and of decomposition. It is possible therefore for the rate of formation of  $C$  to remain constant, increase or decrease with time, and there is not necessarily any simple relation between the concentration of  $A$  and the rate of formation of  $C$ .

#### SUMMARY.

1. The velocity of hydrolysis of gelatin by trypsin increases more slowly than the gelatin concentration and finally becomes nearly independent of the gelatin concentration. The relative velocity of

hydrolysis of any two substrate concentrations is independent of the quantity of enzyme used to make the comparison.

2. The rate of hydrolysis is independent of the viscosity of the solution.

3. The percentage retardation of the rate of hydrolysis by inhibiting substances, is independent of the substrate concentration.

4. There is experimental evidence that the enzyme and inhibiting substance are combined to form a widely dissociated compound.

5. If the substrate were also combined with the enzyme, an increase in the substrate concentration should affect the equilibrium between the enzyme and the inhibiting substance. This is not the case.

6. The rate of digestion of a mixture of casein and gelatin is equal to the sum of the rates of hydrolysis of the two substances alone, as it should be if the rate is proportional to the concentration of free enzyme. This contradicts the saturation hypothesis.

7. If the reaction is followed by determining directly the change in the substrate concentration, it is found that this change agrees with the law of mass action; *i.e.*, the rate of digestion is proportional to the substrate concentration.

Most of the experimental work in this paper was done by Mr. Frank Johnston.



## THE INFLUENCE OF X-RAYS ON THE PROPERTIES OF BLOOD.

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In the course of an experimental investigation concerning the nature of the reaction of the animal organism to x-rays we have made observations which we believe to be of sufficient interest to communicate in the form of a preliminary report. Our experiments were originally designed to throw some light on the question raised by the statement made by some roentgenologists that the characteristic illness frequently observed in individuals after an exposure to massive therapeutic doses of x-rays is due to a state of acidosis. The basis of this statement is apparently an empirical one since there is no rational evidence to support such an idea. We do not interpret the results of the experiments to be reported in this paper as a final answer to this question. We shall discuss this subject in a later paper.

Our procedure has been to study the numerical changes in the white cells of the blood, and the pH and bicarbonate content of the plasma in rabbits following an exposure of these animals to x-rays. Rabbits were chosen as the most convenient of the usual laboratory animals for our first observations on account of the facility with which they can be bled from the heart. We realize, however, that they are not ideal animals for our ultimate purpose. The plasma was obtained from blood drawn under paraffin oil into a tube which contained a sufficient amount of potassium oxalate to prevent coagulation. From 15 to 20 cc. of blood were drawn and the final oxalate concentration was about 0.3 per cent. Immediately after drawing the blood the plasma was separated from the cells by centrifuging. The surface of the oil was covered with low freezing point paraffin before centrifuging to prevent contact with air. We have found it possible to bleed a rabbit four or five times a week without any apparent effect on the

general reaction of the animal, as regards the conditions of our investigation. For the pH determinations we have employed the colorimetric method developed by Cullen,<sup>1</sup> and for determining the bicarbonate content we have employed the procedure of Van Slyke.<sup>2</sup> The pH determinations have been checked electrometrically as have also the standard buffer solutions used in the colorimetric tests.

Preliminary observations showed the range of the pH value of rabbit plasma to be between 7.12 and 7.26, values which are lower than those stated by Hasselbalch and Lundsgaard (1912), 7.33 (electrometric), and Kuriyama (1913), 7.4 to 7.5 (colorimetric). However, since the electrometric method employed in obtaining our values is more precise than was the method available at the time the values of Hasselbach and Lundsgaard were determined, we believe our values to be more nearly correct. We found the normal plasma bicarbonate content, expressed in volumes per cent of CO<sub>2</sub>, to vary between 26 and 45. It is possible to obtain wide variations in a single rabbit unless careful attention is given to keeping the animal quiet during the process of bleeding. Feeding also plays a rôle in the variations of different samples of plasma but we have found it possible to control this factor by standardizing the amount of food and the time of feeding and the interval of time between the removal of animals from food before the beginning of the experiment.

The rabbits have been exposed to x-radiation from a Coolidge tube, the dosage factors being as follows: spark gap, 3 inches; current, 10 milliamperes; target distance, 6 inches; and time of exposure, 15 minutes. This is a massive dose of radiation of relatively long wave lengths, a large part of which are absorbed by the skin. This dose approaches the maximum amount that can be given without killing the animal and was purposely chosen as a starting point in order to obtain clear cut results. The exposures were made directly on the rabbit's abdomen which had been previously shaved.

#### EXPERIMENTAL.

1. Four rabbits were bled from the heart for plasma, then two of the animals were exposed to the radiation stated. The other two

<sup>1</sup> Cullen, G. E., *J. Biol. Chem.*, 1922, p. xvii.

<sup>2</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

animals were not x-rayed. One control and one test animal were bled again 24 hours later and the other control and test animals were bled 48 hours after the x-ray exposure. 2 weeks later the test animals were bled again. The observations made on the plasma obtained at these times are given in Table I.

TABLE I.

Rabbit No.	Before radiation.		24 hrs. after radiation.		48 hrs. after radiation.		14 days after radiation.	
	pH	CO <sub>2</sub>	pH	CO <sub>2</sub>	pH	CO <sub>2</sub>	pH	CO <sub>2</sub>
		<i>vol. per cent</i>		<i>vol. per cent</i>		<i>vol. per cent</i>		<i>vol. per cent</i>
1 (x-rayed.)	7.16	33.2	7.34	51.0			7.22	51.2
3 "	7.21	35.6			7.35	49.6	7.23	48.6
2 (Normal.)	7.20	35.2	7.15	33.5				
4 "	7.19	38.6			7.14	34.7		

This experiment was repeated with results similar in magnitude to those given.

2. Two rabbits were bled then x-rayed as stated. Blood was obtained from one of them an hour after the x-ray exposure, and from the other 3 hours after. The results of the observations on the plasma obtained are given in Table II.

TABLE II.

Before radiation.		1 hr. after radiation.		3 hrs. after radiation.	
pH	CO <sub>2</sub>	pH	CO <sub>2</sub>	pH	CO <sub>2</sub>
	<i>vol. per cent</i>		<i>vol. per cent</i>		<i>vol. per cent</i>
7.26	38.5	7.30	48.0		
7.24	38.8			7.35	54.6

In other experiments observations have been made on the plasma at various intervals following exposure to the x-rays and the above results corroborated.

Blood counts have been made on rabbits which were bled as well as x-rayed, and on others which were x-rayed only the changes do not differ significantly. When counts are made at 15 minute intervals the first decrease we have noted on the mononuclear elements occurs

$\frac{1}{2}$  hour after the x-ray exposure. After the first hour the interval between counts has been 1 hour and the maximum decrease occurs between 3 and 5 hours, the same period noted for the maximum change in the reaction of the plasma.

Summarized briefly the results of these experiments show that there exists a definite alkali excess in the body of the rabbit following an exposure to the dose of x-rays employed. Since there is an increase in the bicarbonate content of the plasma as well as an increase in the pH (*i.e.* a decrease in the  $C_{H^+}$ ), then evidently the  $\frac{BA}{HA}$  ratio must be

disturbed in a manner which defines a state of uncompensated alkali excess.<sup>3</sup> The maximum change in the reaction of the plasma is reached between 3 and 5 hours following exposure to radiation. The pH is observed to reach a normal level before the bicarbonate, which indicates the existence of a period of compensated alkali excess before the animal returns to normal. Evidence is found which significantly associates the point of maximum change in the reaction of the plasma with the maximum change noted in the white cell counts of the blood.

With these facts established we directed our attention to the possibility of producing a state of alkali excess in the rabbit by injecting sodium bicarbonate, and to the study of any chemical and morphological blood changes which might ensue. As a guide to the amount of salt to inject, we availed ourselves of the formula developed by Palmer and Van Slyke,<sup>4</sup> for bicarbonate administration with reference to the effect of a given amount of salt on the reaction of the body fluids. The formula is based on the fact that 1 gm. of  $NaHCO_3$  yields 267 cc. of  $CO_2$  measured under standard conditions. If now it is assumed that 0.7 of the body weight is fluid, it is obvious that there are 700 cc. of fluid for each kilo of body weight and 1 gm. of bicarbonate would increase the  $CO_2$  content  $\frac{267}{7W}$  volumes per cent. This expression simplifies to  $\frac{38}{W}$  where  $W$  is the body weight expressed in kilograms. If now we let  $b$  = the volume per cent increase in  $CO_2$ , we

<sup>3</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlviii, 1.

<sup>4</sup> Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.



have the simple equation  $b = \frac{38}{W}$ . Obviously for  $g$  gm. of  $\text{NaHCO}_3$  the equation becomes  $g = \frac{bW}{38}$ . As our results show, this equation permits one to calculate with a good degree of accuracy the change to be expected.

Four rabbits were bled and the bicarbonate content and pH of their plasma was determined. Later they were given an intraperitoneal injection of sodium bicarbonate in amounts stated and the reaction of their plasma was again determined after an interval of 1 hour with the results given in Table III.

TABLE III.

Rabbit No.	Weight.	NaH CO <sub>3</sub> injected.	Before injection.		1 hr. after injection.			Difference between calculated and observed CO <sub>2</sub>
			pH	CO <sub>2</sub>	pH	CO <sub>2</sub> observed.	CO <sub>2</sub> calculated.	
	<i>kilos</i>	<i>gm.</i>		<i>vol. per cent</i>		<i>vol. per cent</i>	<i>vol. per cent</i>	<i>vol. per cent</i>
1	2.3	?	7.14	36.2	7.30	67.0	69.2	-2.2
2	2.3	3	7.19	35.6	7.42	88.1	85.2	+2.9
3	2.2	1	7.17	37.6	7.35	52.2	54.9	-2.7
4	2.1	1	7.22	40.6	7.36	55.4	59.9	-4.5

In other experiments similar observations have been made at varying intervals following injections of 1 gm. of bicarbonate. All injections were made into the peritoneal cavity. For periods after 1 hour the observed values are much less than the calculated values. The pH returns to normal in about 3 hours, but the bicarbonate content remains high up to 5 hours.

Observations on the white blood cells of these rabbits, as well as on other rabbits similarly treated show a diminution of white blood cells per cc. and the absolute counts show this to be due largely to the mononuclear forms. The maximum effect is noted 1 to 3 hours after the injection of the salt which time coincides with the period of maximum change in the reaction of the blood. This is true also in the rabbit following exposure to x-rays. Immediately following this maximum decrease there is a continued rise in the number of white blood cells per cc. and a rapid return to normal. It is possible, however, to maintain a low level of the mononuclear cells by repeated

injections of the salt. This is true in the guinea pig too, the only other animal to which we have so far extended these observations. Following an injection of 5 cc. of physiological saline solution we observe no significant change in the blood picture compared with blood counts on normal animals made at similar intervals.

#### DISCUSSION.

The facts thus presented develop an interesting analogy between the changes associated with x-ray effects and those found following injections of sodium bicarbonate. We do not feel justified at this time in attempting to make a definite interpretation of these results. The analogy, however, seems to be a striking one and it is very suggestive for further study, since it presents a possible opening for investigations concerning the mechanism of x-ray action on the animal body. Furthermore, evidence is found which justifies a doubt that the characteristic cell changes noted following x-ray exposures are due to the direct action of the x-rays. Additional force is given this doubt by the results of further experiments now in progress in which we are observing the effects accompanying the injection of other substances. So far we have used sodium chloride, sodium sulfate, monosodium phosphate, and glucose. Very definite changes are noted in the blood picture, but the data we have to date are not sufficient to permit of any further statement.

#### CONCLUSION.

When rabbits are exposed to x-radiation as described, there results a definite change in the  $\frac{BA}{HA}$  ratio of their plasma. This change is evidently one which defines a state of uncompensated alkali excess.

The time required for a maximum change in the chemical reaction is the same as that required for a maximum change in the decrease of leucocytes.

Sodium bicarbonate injected into the peritoneal cavity is followed by results identical with those observed following exposure to x-rays. The maximum changes occur in a shorter time following bicarbonate injection, but the relation between chemical and morphological changes are the same.

We consider this analogy to be an important one in that it is suggestive of a relationship between the effect of salt and the effect of x-rays.

# KINETICS OF THE BIOLUMINESCENT REACTION IN CYPRIDINA. I.

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## INTRODUCTION.

Until the present time it has been proven impossible to isolate as pure chemical individuals any of the reactants concerned in the production of light by organisms. The work of Dubois<sup>1</sup> and Harvey has, however, indicated the general nature of such reactions as consisting in the oxidation of a substrate, luciferin, catalyzed by an enzyme, luciferase. Harvey<sup>2</sup> believes that luciferin should be placed among the natural proteoses on the borderland between the proteoses and peptones, whereas luciferase is a protein probably belonging to the albumin group.

Quantitative studies upon such reactions are beset with several difficulties, among the most important of which are (1) absolute lack of information as to the concentration of the reactants present in extracts, and (2) the rather highly labile character of the substrate, luciferin, which readily oxidizes spontaneously without light production in the absence of the enzyme. I have made an attempt, however, to discover something of the time relations involved in bioluminescent reactions, specifically to study the rate of decay of the light produced when aqueous solutions of enzyme and substrate are added together. This paper deals with a photographic method which has been developed for this study, and with the results which it indicates.

<sup>1</sup> Dubois, R., *La vie et la lumière*, Paris, 1914.

<sup>2</sup> Harvey E. N., *J. Gen. Physiol.*, 1919, i, 269.

*Material.*

The material used throughout the study has been the Japanese ostracod, *Cypridina hilgendorffii* Müller. These animals produce one of the brightest, if not the brightest, animal light reactions, which has yet been described. According to Nichols,<sup>3</sup> the maximum brightness of the luminescence in this form is 14.5 to 16 millilamberts, a value higher than that for any fluorescent preparation except some of the uranyl double sulfates. Coblenz has found that a tungsten filament at 2,000°C., that is at about the temperature of ordinary incandescent lamps of the vacuum type, has a brightness of 630,000 millilamberts. *Cypridina* light, therefore, while well up in the scale of brightness for luminescences, is still comparatively faint.

The *Cypridinae* have been placed at my disposal in generous quantities by Dr. E. N. Harvey. They have been dried, powdered, and ether extracted, and have furnished a convenient and highly satisfactory material. Aqueous solutions of the enzyme, luciferase, are prepared by extracting *Cypridina* powder with cold water until the luciferin has been completely oxidized, and light has ceased to appear. I have made it a constant practice to extract 5 gm. of the powder with 50 cc. of distilled water. This does not secure a standardization of enzyme strength, for the enzyme, as well as the luciferin, deteriorates with time, although not as rapidly as the latter. It does permit of a measure of control of reaction velocities, producing enzyme strengths of the same order of magnitude. Samples thus extracted are filtered, and the filtrate is used within the following 3 or 4 days. Since chloroform and other preservatives hasten the deterioration of such extracts, I have usually used freshly prepared luciferase to which no chloroform has been added.

Luciferin solutions are prepared fresh for each experiment by the extraction of a sample of the dry powder with boiling water. I usually extract about 2 gm. with 40 cc. of water. After the addition of the water, boiling is continued for a few seconds to destroy the enzyme completely; the solution is then rapidly cooled under the tap. The resulting yellowish solution is allowed to stand for about

<sup>3</sup> Nichols, E. L., *Science*, 1922, lv, 157.

5 minutes until the extracted particles have settled out well. The clear supernatant solution is then carefully decanted and used as needed. I have not attempted to filter such solutions, since I early found that the time used, and exposure to air required, brought about a great diminution in luciferin concentration, due to the spontaneous oxidation already mentioned. This spontaneous reaction has entered in at various points to complicate the problem. It will later be considered in more detail.

### *The Photographic Method.*

Many students of the physics of light have interested themselves in the kinetics of the true inorganic phosphorescences. For a discussion of this field and references to its extensive literature the reader is referred to the memoir of Nichols and Merritt.<sup>4</sup> Direct photometric methods have been universally employed in such work. No previous studies of a similar sort on the time relations of either chemiluminescent or bioluminescent reactions have been reported. In the present work I have attempted to photograph the reaction, by developing a method of photographic photometry.

At first glance it might appear that in such a problem a direct photometric study is feasible and advisable. I was early convinced by a number of considerations that this was not the case in an animal light reaction. The rapidity of the decay in such aqueous solutions as seemed best for study, together with the low intensities of light involved, would make it very difficult, if not impossible, to follow the reaction in this way. The final, and absolutely determining consideration, was that for such studies as those involving the effect of concentration of enzyme and substrate, which I wished to attempt, it would be necessary to investigate two solutions simultaneously. This simultaneous study is necessitated by the fact of rapid deterioration in luciferin solutions, already referred to, so that successive studies of two originally identical portions of such a solution could not possibly give comparable results. A photographic method presented the only possibility for such simultaneous studies.

<sup>4</sup> Nichols, E. L. and Merritt, E., Studies in luminescence, *Carnegie Inst. Washington, Pub. No. 152*, 1912.

Photographic methods for the study of various bioluminescences have been developed by several investigators. Friedberger and Doepner<sup>5</sup> employed a photographic method of determining the intensity of the light coming from a culture of luminous bacteria. They impressed upon their plates a calibration exposure made with a standard lamp of known intensity, and evaluated the densities with the Martens polarization photometer. It would appear that they did not attempt to control the quantity of their calibration light source so as to match that of the organic luminescence in question. Probably the most careful and important use of photographic photometry in such a study is the work of Ives and Coblenz<sup>6</sup> who worked out the spectral energy curve for the firefly, evaluating the intensities along the spectrum by comparing the photographic densities produced by the light of the animal with the photographic effects of a carbon glow lamp, whose spectral energy distribution had been accurately determined.

In the present investigation I have used throughout Eastman cine-negative motion picture film. The spectral sensitivity of this film extends to about  $\lambda = 0.59\mu$ . This film was obtained from the company before perforation so that the whole width of the film is available for use. The variation in density from point to point in a film, produced by identical exposures, is somewhat less than in plates, in which emulsions are liable to greater variations in thickness. The Eastman laboratories have informed me that the variations to be expected in the developed densities of this film are an average of 3 per cent, and an extreme of 6 per cent. Intensity values calculated from such density readings will vary by somewhat higher percentages. Such deviations are of course unfortunate, but represent a difficulty inherent in all methods of photographic photometry. It must be recognized that great precision is not possible with any photographic method. In the present work I have been able to assure myself that I was working well within the expected error of the film; all records have indicated that this was the case. Every

<sup>5</sup> Friedberger, E., and Doepner, H., *Centr. Bakt., 1te Abt.*, Orig., 1907, xliii, 1.

<sup>6</sup> Ives, H. E., and Coblenz, W. W., Luminous efficiency of the firefly, *Bull. Bureau of Standards*, 1909, vi, 321. Also see Coblenz, W. W., A physical study of the firefly, *Carnegie Inst. Washington, Pub. No. 164*, 1912.

effort has been made to control the whole manipulation of this film so as to secure the greatest possible uniformity. To facilitate this end only short strips of film have been used, in which great variations in the emulsion would not be expected. The standard length of record finally adopted is 205 mm. long. The whole length of film used, including the records of the reaction, and the calibration exposures, is 250 mm.

After several unsuccessful attempts to construct some optical system which would function to focus the light given out by the reaction upon a moving film, I turned to the use of a simple test-tube container to the side of which is cemented a phosphor-bronze strip in which a vertical slit window has been cut. The two containers used throughout the work were carefully chosen to be of exactly the same bore. The two slit windows were cut in the phosphor-bronze strips by a milling machine, to a width of 1.5 mm. and a height of 6 mm. In width they differ by an error of considerably less than 1 per cent. The two bronze plates are cemented to the containers in identical positions, and were so applied that absolutely clear glass only is apposed to the slit openings. The inner edges of each slit come flush with the glass of the container. All outer surfaces of both tube and brass plate are painted a flat black to prevent transmission or reflection of light. Light can pass from the containers only through the slit windows themselves. The inner edges only of these openings are unpainted, since I wished to avoid the introduction of large errors in slit widths which might arise in the application of such coverings.

With this simple arrangement it is possible to follow the decay of light intensity in reacting *Cypridina* solutions with considerable accuracy. The two containers, mounted upon horizontal supporting bars, adjustable in length, are clamped rigidly upon uprights, and are brought close to the drum of a Zimmermann kymograph. They are adjusted for each experiment so that the line connecting container center and drum center passes through the middle of the slit window, giving a symmetrical and identical orientation for both containers with respect to the drum. The two containers are placed side by side, one of them being fixed about 10 mm. higher than the other. The slit windows are thus presented to two bands of the drum surface

as it revolves, these bands being separated by a narrower strip some 4 mm. wide. An inspection of Plate 1 will make clear the nature of the records resulting from these arrangements.

Records are taken in a dark room. A length of film is wound tightly about the drum, firmly fastened, and then revolved past the slit windows, where it is affected by light issuing from the reacting solutions placed within the two containers. Records are thus taken simultaneously. The Zimmermann kymograph, whose motion is controlled by a governor, gives quite accurate and constant rotation until the spring has nearly unwound. All records have been made with the spring fully wound up at the beginning of the experiment. No attempt has been made to impress any sort of time record upon the film, in view of the high degree of constancy in the speed of rotation. Since absolute measurements of time are of no importance, I have made it a practice to read time in millimeters along the film, and all records presented in this paper are graphed with such values as abscissæ. The actual time of rotation of the drum has been 2 minutes, and the actual speed of the film 4.123 mm. per second.

The containers are brought to a standard distance of 1 mm. from the surface of the drum. This leaves a narrow space through which, in darkness, the film strip can be wound into position about the drum. Under these circumstances there is little diffusion to the sides from the slit windows, but a narrow region to right and left is affected by such diffuse light, and this is undoubtedly a factor affecting the records. Because of it, and of the slit width itself, the effect of the light upon any particular halide grain in the revolving film is not instantaneous. Allowing for such diffusion the exposure time for any particular grain is about half a second. No attempt was made to correct mathematically for these factors of slit width and diffusion, since their effect upon the shape of the curve is slight in any case, and indeed tends to smooth the curve rather than to distort it.

With the containers correctly adjusted, and the film applied to the drum, aqueous luciferin solutions are added to both vessels. I have made it a uniform practice to use 20 cc. of luciferin solution in each container, measured out with a pipette. To each solution, at the proper instant, while the drum is in rotation, there is added 1 cc. of luciferase. The two enzyme solutions have previously been



taken up in a double pipette controlled by a single bulb. Pressure upon this bulb throws an enzyme solution into each tube at exactly the same instant, and with considerable and identical force. In some few experiments I have relied upon the agitation thus produced to secure mixing, and the resulting records have given every evidence of uniformity and consistency. In order, however, after the usual chemical procedure, to insure thorough and continuous mixing of the reactants, I have in most cases stirred both tubes by means of two small motor driven propellers, run at identical speeds, and dipping just below the surface of the reacting solutions. The blades are arranged to throw the surface film continually into the depths. The oxygen supply is thus also replenished, but, as will be seen below, this factor does not appear to affect the reaction velocity.

Eastman cine-negative film is 35 mm. wide. The lower record 6 mm. wide, runs parallel to the lower edge of the film, and at a distance of some 4 mm. from it, the upper record, of the same width, runs through the middle of the film, its lower edge at 4 mm. distance from the lower record. The upper third of the film is thus left blank, and upon it later there is impressed a series of calibration exposures, shortly to be considered.

It can be easily seen that in a photographic study in which quantitative measurements are desired, an accurate knowledge of the relation between the intensity of the incident light and the resulting photographic density is essential. I have made no attempt to secure information of this sort in any absolute terms, although such data would undoubtedly be most interesting if it could be readily obtained. Certain difficulties have made impossible the gaining of such absolute determinations. The whole study has been one of purely relative values.

A host of studies upon the physics and chemistry of the photographic process have given rather extensive information as to the intensity-density relationships. Those who are interested in a detailed discussion of this subject may consult the book of Sheppard and Mees.<sup>7</sup> The curve of blackening of a film or plate assumes a characteristic elongated S-shaped form when the logarithms of the

<sup>7</sup> Sheppard, S. E., and Mees, C. E. K., *Investigations on the theory of the photographic process*, London, New York, Bombay, and Calcutta, 1907.

intensities of the incident light are plotted as abscissæ, and the resulting densities as ordinates. The term density is defined as the logarithm of the opacity, while the opacity of a film or plate is itself the reciprocal of the transmission. In the determination of photographic densities, therefore, it becomes necessary to secure a method for measuring transmission values; that is, to determine what percentage of a given constant light is able to pass through the density in question. Various methods for such studies have been perfected.

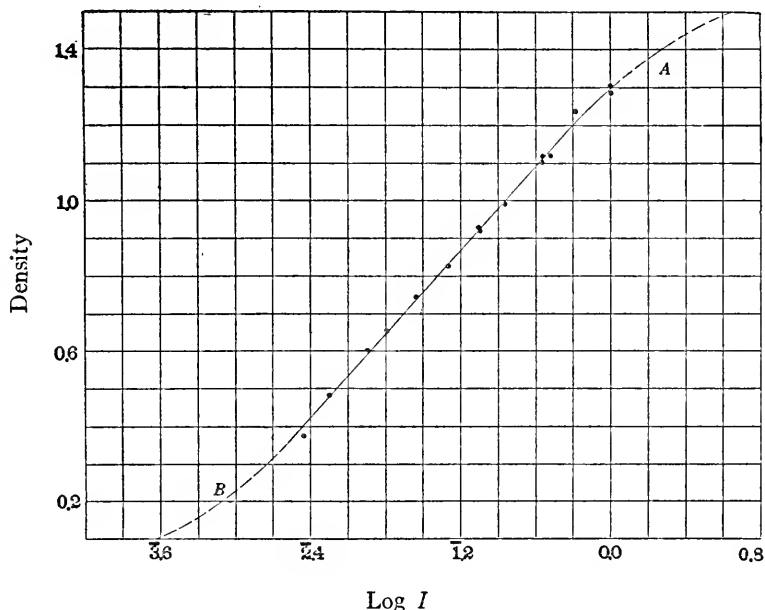


FIG. 1. Curve of blackening. Straight line portion determined by points obtained with the calibration method using *Cypridina* light itself as the light source. Dotted lines indicate diagrammatically how the over-exposure (A) and under-exposure (B) segments would appear. Abscissæ represent logarithms of light intensities (filter transmissions). Ordinates represent photographic densities.

Perhaps the most popular has been the polarization photometer of Martens. In my own work I have employed the optical pyrometer, a photometric instrument of great accuracy.

The nature of the curve of blackening can be best understood by reference to Fig. 1, which will also indicate how closely it is possible

to connect light intensity with film density. The dotted portions of the curve represent roughly the two ends of the S, which are not determined by the experimental values obtained in this particular calibration exposure. It will be seen that the curve follows a straight line along a great part of its course, that part, indeed which is determined by the experimental values. This straight line portion of the curve is often spoken of as the normal exposure segment. The upper shoulder of the curve forms the over exposure, the lower heel the under exposure segment. I have endeavored so to control all of the experiments as to hold both moving record and calibration densities to the normal exposure region, although occasionally the densities run out upon the upper shoulder of the S in their higher values.

In photographic literature the contrast of a plate is called gamma ( $\gamma$ ). It may be defined as the rate of growth of density with the logarithmic increase of exposure. Graphically it is the slope of the straight line segment of the curve of blackening. The shape of this curve, and especially the slope of the straight line segment, are affected by many factors of which time and temperature of development, kind of developer employed, and the quality of the previously incident light are the most important. In view of the changes thus effected in this curve it is always highly desirable to impress calibration densities upon each individual record, so that such calibrations may progress through all the vicissitudes of development side by side with the regions of the film affected by the unknown intensities of light which are to be evaluated. The two series are then strictly comparable. The paper by Jones<sup>8</sup> (1920) indicates the magnitude of the changes in the value of the gamma produced by developmental variations.

The quality of the incident light affects the slope of the curve of the blackening to some extent, although there appears still to be some dispute among students of the subject as to the magnitude of this effect. Shepherd and Mees (1907) observed differences in the shapes of blackening curves obtained with red and violet light. Some few workers have claimed that gamma does not change at all with different

<sup>8</sup> Jones, L. A., *J. Franklin Inst.*, 1920, clxxxix, 469.

wave lengths (Leimbach,<sup>9</sup> 1909). Abney,<sup>10</sup> Stark,<sup>11</sup> Helmick,<sup>12</sup> Ross<sup>13</sup> and others report more or less marked differences. The recent paper of Ross<sup>13</sup> (1920) is of interest in this connection. His results indicate a very considerable variation, and are of particular interest since they cover in some detail the region of the spectrum in which the *Cypridina* luminescence falls. Thus with identical exposure and development the following values for gamma were obtained:  $\lambda = 4100\text{\AA}$ ,  $\gamma = 0.74$ ,  $\lambda = 4400\text{\AA}$ ,  $\gamma = 1.15$ ,  $\lambda = 4800\text{\AA}$ ,  $\gamma = 1.42$ ,  $\lambda = 5100\text{\AA}$ ,  $\gamma = 1.04$ . In the light of such data it will be seen that the effect of the quality of the light is indeed considerable.

It is therefore plain that for the evaluation of photographic records produced by unknown light intensities it is of prime importance that the calibration exposures should be produced by light of exactly the same quality. The spectral distribution of light in *Cypridina* luminescence was studied by Harvey<sup>14</sup> (1920) who reports that visual spectroscopic observation indicates a broad band spectrum extending from  $\lambda = 4150\text{\AA}$  to  $\lambda = 6100\text{\AA}$ , the broadest distribution yet observed for any bioluminescence. The light of *Cypridina* is therefore decidedly heterochromatic, and its photographic effect is a composite of the effects of all the different wave lengths included in the light. The spectral energy curve has not yet been worked out for *Cypridina*, so it is at present quite impossible to know the relative weights of the different colors involved in producing the composite effect. It is, moreover, impossible to know how to match this photographic effect exactly by using a light filter and a standard lamp, although such a match might conceivably be obtained through a study of the effects of many filters. I early decided that it would be decidedly questionable to employ such a colored filter-standard lamp method of calibration, in the lack of accurate information concerning the energy

<sup>9</sup> Leimbach, G., *Z. wiss. Photo.*, 1909, vii, 157.

<sup>10</sup> Abney, W. W., *Treatise on photography*, London, New York, Bombay, and Calcutta, 10th edition, 1901, 413.

<sup>11</sup> Stark, J., *Ann. Phys.*, 1911, xxxv, 474.

<sup>12</sup> Helmick, P. S., *Phys. Rev.*, 1921, xvii, 145.

<sup>13</sup> Ross, F. E., Communication No. 95 Research Laboratory, Eastman Kodak Company. Also in *Astrophys. J.*, 1920, lii, 98.

<sup>14</sup> Harvey, E. N., *The nature of animal light*, Philadelphia and London, 1920, 47.

distribution of the *Cypridina* luminescence. I have sought to achieve the desired results in another way.

The method finally adopted makes use of the *Cypridina* light itself for the impressing of calibration exposures. Two-thirds of the width of the film strip is given over to the moving records. Upon the other third there is impressed a series of fifteen calibration exposures. Plate 1 illustrates several typical records which indicate the results of this method.

The fifteen calibration exposures are impressed simultaneously through a series of fifteen windows in which are placed neutral photographic filters of various known transmission values. Measurement by the pyrometer has indicated that the filters used are very nearly neutral throughout the blue and green regions of the spectrum; that is, throughout that region in which *Cypridina* light affects the film. Absolute neutrality in such screens is impossible of attainment.

The series of filters are mounted in windows cut in a brass strip, wide enough to shield the whole width of the film, and bent in the form of the arc of a circle with a radius of 15 mm. (Fig. 2). At the center of this circle a test-tube 25 cm. in diameter is mounted. The test-tube is painted a flat black with the exception of a segment, *A*, which gives a ring of light at the level of the windows, when reacting *Cypridina* solutions are placed in it. After the moving records of the luminescence have been impressed upon the lower two-thirds of the film, the latter is clamped tightly between the brass strip, *B*, carrying the filters, and a rigid back, *C*, bent to the same curvature. Fifteen spots on the unexposed third of the film are thus brought opposite to the fifteen openings in strip *B*, all spots being equidistant from the ring opening in the test-tube at the center. A concentrated luciferin solution is placed in the test-tube, a motor driven propeller sets the whole solution into rapid rotation, and a concentrated enzyme solution is added. The resulting illumination of the ring opening *A* presents an absolutely identical light source to all of the fifteen windows simultaneously. This illumination is indeed not constant, but falls rapidly through the typical curve of decay characteristic of the reaction. This rapidly diminishing light source, however, exerts an identical effect in every direction at each instant, and the only differential factor in the light which reaches the fifteen spots

of film is the absorption of various known percentages of light by the filters. The method integrates upon the film the effect of a rapidly changing, but always homogeneously radially distributed, light source, over a sufficient length of time to produce photographic densities lying in the same region of the curve of blackening as those caused by the unknown intensities of the moving records which are to be evaluated. I believe that Fig. 1 will give sufficient indication that this is an altogether feasible experimental method. In no case

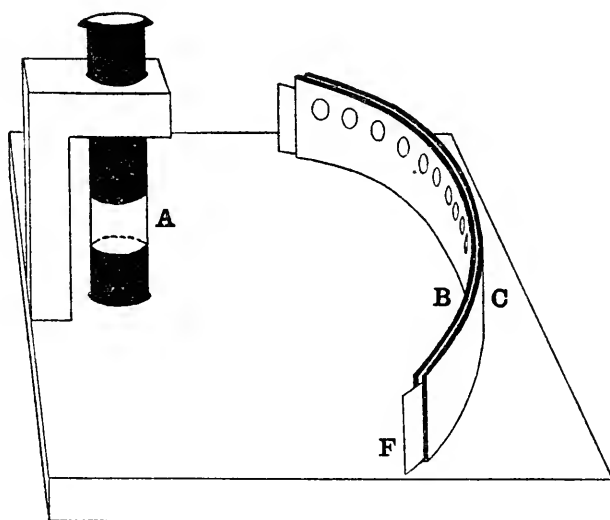


FIG. 2. Diagram of calibration method. The film, *F*, upon the lower two thirds of which the moving records have previously been impressed, is clamped between the rigid back, *C*, and a movable strip, *B*, carrying the fifteen filters of known transmissions. The unexposed third of the film comes opposite to the fifteen openings. Calibration exposures are impressed by light from a luminescent solution ring, *A*, at the level of the filters, giving light with homogeneous radial distribution.

are the density values in error from the mean curve by more than the known error inherent in the photographic material itself. Table I tabulates the known transmission values of the fifteen filters used. Filters 1 to 12 follow a decreasing order of transmission. Filters 13 to 15 give control exposures to check the radial distribution of light.

Windows 1 and 15 carry no filter screens at all, their transmission values being taken as unity. Transmission values for the other windows are expressed as percentages of these two. In practically every experiment the densities produced at 1 and at 15 check well within the known average error of the film.

The whole calibration apparatus is enclosed in a box lined with black felt. All metal parts are painted a flat black, and every precaution has been taken to secure complete absorption of all light

TABLE I.  
*Transmissions of Calibration Filters.*

Filter.	Transmissions.
	<i>per cent</i>
1	100.00
2	65.92
3	48.30
4	43.75
5	26.91
6	20.04
7	13.71
8	9.02
9	6.34
10	5.07
11	3.23
12	2.24
13	19.63
14	42.17
15	100.00

which is not directly incident upon the film through the fifteen openings. There remains only the possibility of cross reflection between the portions of the film exposed through the filters. Calculation has shown that this could not possibly increase the light incident upon other filters by more than 0.5 per cent, and this factor has been neglected.

The determination of the transmission values of filters 2 to 14 requires a brief consideration. The light which falls upon such a screen as is formed by a developed density in a photographic emulsion is transmitted in two ways. A certain percentage passes through by

direct transmission through the spaces between grains. In addition there is a considerable amount of light which is first incident upon the grains themselves, and is then thrown through the screen at various angles by reflection from them. Such additional light forms the "scatter" or diffusion component. The amount of light actually transmitted and available for affecting the film is the sum of these two components thus directly and indirectly transmitted. In the evaluation of such filter transmissions both compounds are automatically taken care of by measuring the percentage of reduction by the filters of the light from a completely diffusing background. For this purpose I have used an opal glass screen illuminated by a 200 watt Mazda lamp run at constant voltage. The filters to be measured are set in immediate contact with this diffusing screen, so that the diffusing surface is in exactly the same position with reference to the filter as is the film at the time of the calibration exposures. The transmission values are then read off with the pyrometer, with which a blue screen is used. It seemed advisable to check the transmission values so determined by some other method in order to be absolutely sure that these values correctly represented the photographic effect. By a photographic method a parallel set of values were obtained which checked the pyrometer determinations within the error of the photographic material. The pyrometer values have been accepted as being probably the more accurate.

The calibration method just described has proven a rapid and satisfactory way of impressing calibration exposures which upon development give a series of densities whose corresponding previously incident relative intensities are accurately known. From these values a curve of blackening similar to Fig. 1 is drawn for each experiment, and densities read along the moving records are referred to the standard curve for evaluation. Extrapolations for short distances in both directions beyond the experimental points are usually used, permitting a range of reading in good records of as much as 100 per cent to 0.5 per cent in light intensity. The average range is about half of this.

In developing films, the records, emulsion side out, are fastened upon a strip of glass of the same length. The whole film is immersed



at the same instant in the developing bath,<sup>15</sup> and rocked back and forth, and up and down at random throughout the whole period of development, to prevent local changes in developer concentrations. I have not attempted to control temperature rigorously, although the temperature of the bath is always about 18°C. Development times are also varied to meet the varying intensities obtained in different experiments. Development is in darkness, with the exception of occasional momentary viewings under the red light.

The time relations of both calibration and moving records have been so controlled as to make the two sets of exposures comparable. It is known that the reciprocity law of Bunsen and Roscoe does not hold for all values of time. It does hold, however, with close approximation if the time is more than a very small fraction of a second, or less than 100 seconds. Exposures along the moving records are for about half a second, while calibration exposures have in no case exceeded 100 seconds. In all records the great burst of light from the calibration tube is over in a very few seconds. The two series of densities may therefore be compared with a good degree of accuracy.

I have entered into some detail in the above descriptions since they appear rather necessary to a complete understanding of the problem, and particularly since the theory and practice of photography in its quantitative relationships is probably little known among biological readers. For the benefit of these readers I will also discuss briefly the methods employed in determining film densities.

### *The Optical Pyrometer.*

The optical pyrometer is a photometer in which the intensity of light emitted from the filament of a small pyrometer lamp is matched against some other light source whose intensity it is desired to measure. Fig. 3 indicates the details of the optical system. In my work I

<sup>15</sup> The following formula was used, diluted with an equal volume of water:

Solution A. Water.....	64	oz.
Hydroquinone.....	26	gm.
Sodium sulfite.....	190	gm.
Solution B. Water.....	42.4	oz.
Potassium carbonate.....	152	gm.
Potassium bromide .....	2	gm.

have used at *A* a special tungsten wire lamp kindly loaned to me by the Nela Research Laboratories. The lamp has a filament made of a single loop of tungsten wire whose diameter is 0.2 mm. An image of a small vertical portion of this filament is focussed by a lens, *B*, upon the filament of a smaller pyrometer lamp, *C*, enclosed in a housing, and the image of both filaments is then viewed through a telescope at *D*. Both lamps are run from storage cells, the amperage being controlled by variable resistances. The intensity of the larger or background lamp is held at a constant value by continual observation of an ammeter in the circuit. The current through the pyrometer lamp is read from a millimeter in this circuit. The relation between intensity of light in the pyrometer filament and the current producing it is determined by reducing the intensity of the constant background by the interposition of various screens or similar devices of known

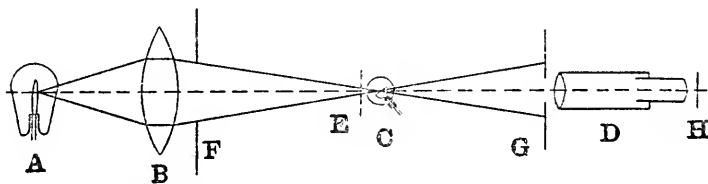


FIG. 3. The optical pyrometer. *A*, background lamp; *B*, objective lens; *C*, pyrometer lamp; *D*, telescope; *E*, film; *F* and *G*, diaphragms; *H*, monochromatic filter.

transmission values. For this purpose I have used the set of sector discs made by the Leeds and Northrup Company. These discs have transmission values of 80, 70, 60, and 50 per cent. At my request the manufacturers made a fifth special disc with a transmission value of 55 per cent. By these discs and combinations of them it is possible to reduce the light to the following percentages of the original value, 80, 70, 60, 55, 50, 35, 30, 25, 20, 15, 10, 5. By reducing the background current two overlapping series of readings connecting the brightness of the pyrometer filament with the current producing that brightness are obtained, extending the range of the calibration to lower transmission values. In each case a disc is rapidly revolved between the background and pyrometer lamp, and the current through the latter is adjusted until the brightness of its filament matches that

of the reduced background. I use a green glass filter at the telescope eye-piece for all density readings, except in the measurement of the calibration filter densities where a similar blue glass screen has been employed.

It is a frequent custom in measuring photographic densities to use a diffusing background similar to that described above for the measurement of the filter transmissions. It is more convenient and equally satisfactory to mount the films to be read in an adjustable holder placed immediately in front of the opening in the pyrometer housing at *E*. Any desired part of record or calibration densities can be interposed between background lamp and pyrometer filament. In this position of the film the light from the background filament is very nearly in focus, the actual focus being about 3 cm. further back at the pyrometer filament itself. The lens and screen at *F* have been so arranged that the image of the filament at the film is 1 mm. wide, and hence a single density determination is made for a strip of film of similar width. At the same time the film is just enough out of focus to prevent the appearance of the developed grains in the telescope, and to give the background at all times a perfectly homogenous brightness.

The transmission values thus obtained represent only the reduction in intensity in the directly transmitted beam. The diffusion component is largely lost. As I wished to compare only one series of photographic densities with another, I have disregarded this diffused light loss. Obviously it makes no difference how such density determinations are made, if the two sets of readings are made in an identical manner. The densities obtained by this method give higher values than would be the case if a diffusing background had been employed. The accuracy of evaluation of the unknown intensities is not affected.

In reading records density determinations have usually been made at every 10 mm. along the film. Such values are calculated from the average of the results of a pair of readings at each point. The permanent photographic record permits rechecking of doubtful determinations. Re-readings usually check to within 1 milliamperes with the original readings. Density values can be determined with great accuracy by this method. The most extensive source of error,

therefore, remains the unavoidable errors inherent in any photographic material.

The optical pyrometer is a standard physical instrument and has a considerable literature. Readers interested in fuller discussions of its theory and practice should consult the papers of Forsythe.<sup>16</sup>

#### EXPLANATION OF PLATE 1.

Reproductions of actual photographic records. The bands are the moving records; the fifteen spots the calibration exposures.

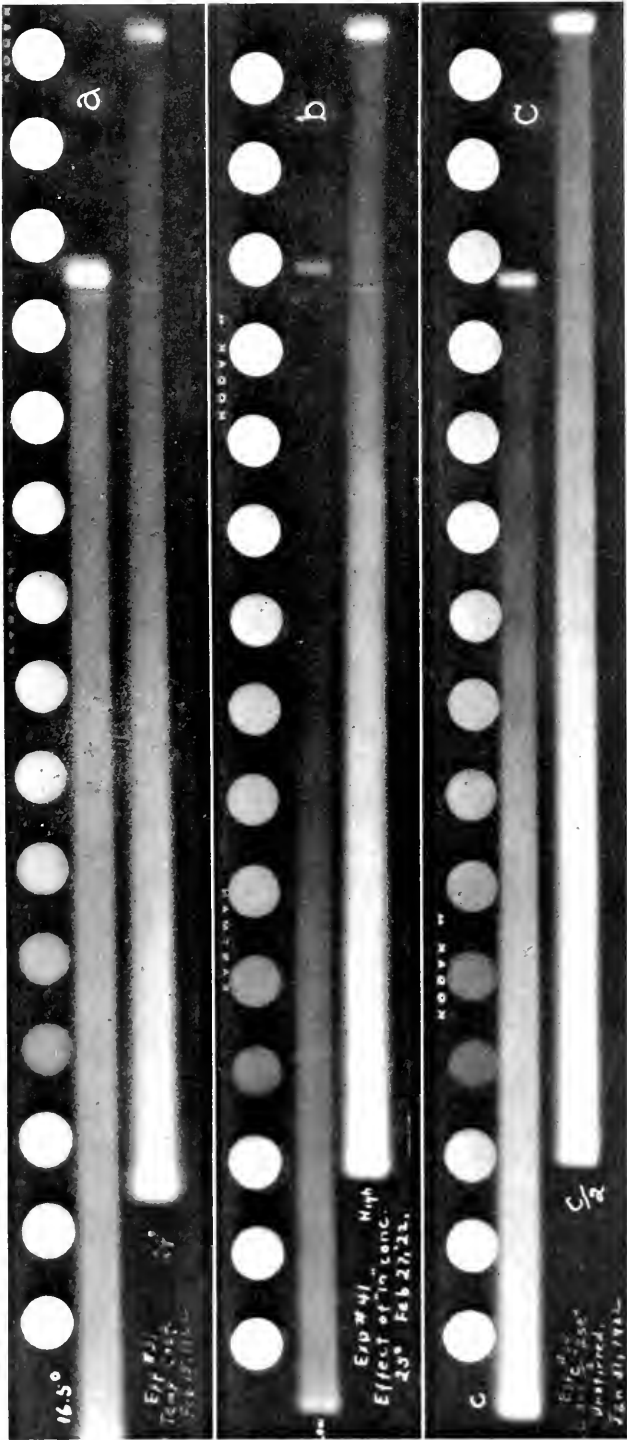
(a) Effect of temperature. Temperatures, 16.5°C. and 24°C.

(b) Effect of luciferin concentration, the low concentration having been obtained by a brief heating of that solution to 55°C.

(c) Effect of luciferase concentrations,  $C$  and  $\frac{C}{2}$ .

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<sup>16</sup> Hyde, E. P., Cady, F. E., and Forsythe, W. E., *Astrophys. J.*, 1915, xlii, 294; Forsythe, W. E., 1919, xlix, 237; *Am. Inst. Min. and Met. Eng., Bull. No. 153*, 1919, 2547; *J. Op. Soc. Am.*, 1920, iv, 305.



Amberson, K. Effect of the volumetric heat reaction in capillary I.



## KINETICS OF THE BIOLUMINESCENT REACTION IN CYPRIDINA. II.

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### EXPERIMENTAL RESULTS.

#### 1. *Nature of the Curve of Decay in Cypridina Luminescence.*

*The Initial Flash.*—A striking feature, observed in practically every photographic record which I have taken, is the occurrence of a very bright initial flash of light, produced at the instant of the union of luciferase and luciferin solutions, and lasting usually for not more than 2 mm. of film, or half a second. An inspection of the plate will indicate the nature of this flash in terms of its photographic effect. The observed initial light intensities in two good typical records are shown in Tables I and II. These values are to be contrasted with the calculated values in the parallel column, which would be consistent with intensity values read from 10 mm. on to the end of the record, which are connected by a simple mathematical relationship soon to be discussed. The initial flash is shown graphically in Fig. 1, where it is indicated by the dotted line at the beginning of the curve.

The significance of this flash is problematical. I have been unable to detect any law by which its magnitude may be predicted. I have very often obtained a practical identity in the values for the initial readings on two simultaneous records whose later courses are quite dissimilar. Yet this is by no means a constant observation, and may have no special significance. It should be recalled that the general practice of the physical chemist, in the study of chemical kinetics, is to allow any reaction a brief space of time at least, to straighten out, before measurements are begun.

*The Law of Decay.*—In an attempt to follow the kinetics of a chemical reaction by the measurement of light intensities emitted

along its curve, it is fundamental to inquire what relation exists between reaction velocity and the resulting light intensity. Trautz<sup>1</sup> from an extensive study of chemiluminescent reactions, but without actual quantitative data, arrived at the conclusion that light intensity is proportional to the velocity of reaction, and therefore a direct measure of it. He based his conclusions upon the observed increase of light intensity in such reacting solutions with the increase of temperature, and upon other similar considerations. Such qualitative observations are by no means adequate to establish such a quantitative hypothesis, but certainly point in the direction of its truth.

In the related field of true inorganic phosphorescence the most comprehensive theory which has yet been proposed is that of Wiedemann and Schmidt,<sup>2</sup> which, in its most general form, supposes that some chemical or physical change is produced in a luminescent material while the exciting radiation is impinging upon it, and that the luminescence which persists when excitation ceases is an expression of the gradual restoration of the original state with the emission of light. More specifically it is generally believed that the effect of the exciting radiation is to split a portion of the luminescent material into equal members of positive and negative particles, probably ionic in nature, and that the luminescence which appears is due to the recombination of these ions at a definite rate. Accurate determinations of the law of decay of light in such materials have given values which fit in well with this hypothesis, if the emitted light is taken to be a direct measure of the number of recombinations at any instant, for it has been found that if the reciprocal of the square root of the light intensity be plotted against time a straight line will connect all the points, and this is the theoretical expectation if two substances, present in equal concentrations, are combining together under the law of mass action. In other words, true inorganic phosphorescences appear to follow the kinetics of a stoichiometric bimolecular reaction.<sup>3</sup> While the theory was originally proposed to explain the form of the

<sup>1</sup> Trautz, M., *Z. physik. Chem.*, 1905, liii, 1.

<sup>2</sup> Wiedemann, E., and Schmidt., C. C., *Wied. Ann.*, 1895, lvi, 177.

<sup>3</sup> It has been found, however, that this is not true at all temperatures, and that some other power of  $I$  than  $\frac{1}{2}$  may better accord with the experimental observations. See Ives, H. E., and Luckiesh, M., *Astrophys. J.*, 1912, xxxvi, 330.



decay curve, and cannot therefore be considered verified by it, the assumptions are at least the most plausible that can be made at present. The *Cypridina* luminescence is not, however, a phosphorescence but an oxyluminescence.

In the present work I adopted as a working hypothesis this assumption that light intensity is a direct measure of reaction velocity. My

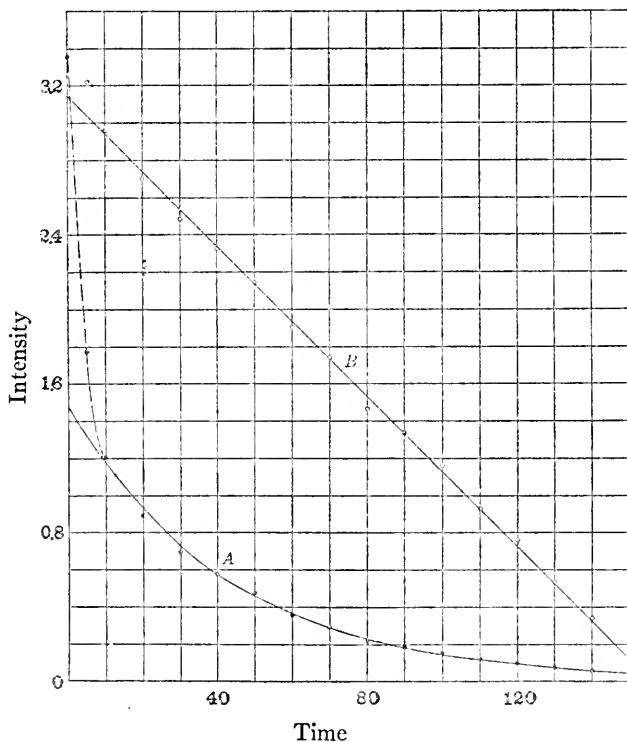


FIG. 1. Decay curve of luminescence in *Cypridina*. *A*. Abscissæ represent time in millimeters along the film; ordinates, intensity of luminescence. *B*. abscissæ represent time; ordinates, logarithm of intensity.

experimental results give a consistent picture when interpreted in terms of such an assumption, and greatly increase the probability of its truth, lending, I believe, quantitative support to the hypothesis of Trautz. Data obtained from a large number of photographic records extending over wide differences in concentration of both

TABLE I.

*Coincidence between Experimental and Calculated Values.*

Time.	Intensity observed.	Intensity calculated.
<i>mm. along film</i>		
0	3.358	1.479
5	1.778	1.318
10	1.202	1.175
20	0.891	0.929
30	0.692	0.731
40	0.575	0.575
50	0.468	0.460
60	0.355	0.367
70	0.295	0.293
80	0.215	0.232
90	0.184	0.184
100	0.152	0.145
110	0.115	0.115
120	0.096	0.092
130	0.073	0.072
140	0.059	0.058
150	0.044	0.046
160	0.035	0.036
170	0.030	0.029
180	0.022	0.023
190	0.018	0.018
200	0.014	0.014

TABLE II.

*Coincidence between Experimental and Calculated Values.*

Time.	Intensity observed.	Intensity calculated.
<i>mm. along film</i>		
0	3.311	1.343
5	1.230	1.130
10	0.975	0.948
20	0.650	0.668
30	0.466	0.472
40	0.331	0.331
50	0.246	0.240
60	0.162	0.170
70	0.105	0.120
80	0.083	0.086
90	0.057	0.060
100	0.041	0.043
110	0.031	0.031
120	0.021	0.022
136	0.016	0.015

enzyme and substrate all indicate clearly that in every case after the first bright flash is over, the emitted light dies out by a simple exponential relationship, so that if the logarithm of the intensity of the light be plotted against time a straight line may be drawn through all the points. A graph of the intensity values obtained in a typical decay curve is shown in Fig. 1, these values being the same as those tabulated in Table I. In *A*, intensity is plotted against time; in *B* the logarithm of the intensity is plotted against time. The exponential relationship is unmistakable. I have found this relationship holding in a large number of separate records, totaling 48 to date. The coincidence between the experimental values and those calculated from the mean curve is not always as good as in the records tabulated in Tables I and II, but is not to be mistaken. I have plotted all other records submitted in the straight line form only, since this form is best adapted to mathematical analysis.

In Tables I and II the calculated values are those taken from the mean curve of the straight line form. The calculated initial intensities are read from the intersection of the straight line on the zero time axis, and are about one-third of the intensities actually recorded in the initial flash.

The form of the decay curve in *Cypridina* is in complete agreement with the theoretical expectation for a monomolecular reaction. For if, according to the standard form,

$$\frac{dx}{dt} = k(A - X)$$

Where  $k$  = velocity constant,  $A$ , initial concentration of the single reactant, and  $X$ , amount of this reactant which has disappeared in the reaction in time  $t$ .

Then by integration

$$k = \frac{1}{t} \log \frac{A}{A - X}$$

$$kt = \log A - \log (A - X)$$

And

$$(1) \quad \log (A - X) = \log A - kt$$

Let  $I$  represent light intensity. Then, by the basic assumption

$$I = \frac{dx}{dt} = k(A - X)$$

Taking logarithms,

$$\log I = \log k + \log (A - X)$$

Substitute for  $\log (A - X)$  its value from (1)

$$\log I = \log k + \log A - kt$$

OR

$$(2) \quad \log I = -kt + \log Ak$$

Equation 2 is the equation of a straight line, in which  $\log I$  and  $t$  are the two variables, and whose slope is negative and proportional to the velocity constant, whose absolute value may be calculated from it, as we shall see. The experimental values plot in agreement with this form, and are interpreted to indicate that the luminescence proceeds as a monomolecular reaction.<sup>4</sup>

From the work of Harvey<sup>5</sup> and others, we can be sure that oxygen is necessary to the progress of the reaction, in addition to the luciferin itself. It may well be asked, therefore, why the concentration of oxygen is not also a determining factor, and why it does not swing the curve over in the direction of a bimolecular form. The answer appears to be that under the conditions of the experiment, oxygen is always present in excess, and that it therefore does not affect the form of the decay curve.

I have made it a practice to read off the slopes of the straight line plottings directly from the graphical form, and have used these values as if they were the velocity constants, since we are here concerned with relative values, and ratios, and not absolute values. All tabulations of relative values of  $k$  have been determined thus directly from the graphs. These values of course depend upon the choice of coordinate scales. The absolute value of  $k$  may easily be calcu-

<sup>4</sup> In a note published from the Nela Research Laboratories (Amberson, W. R., *J. Franklin Inst.*, 1922, cxciii, 111.) I stated that the first results of the study indicated a bimolecular form for the decay curve. I have since found that in the first few records my photographic technique was not satisfactory for good quantitative determinations. At the advice of Dr. L. A. Jones I adopted the development technique used by the Eastman Laboratory in their method of photographic photometry, and have since obtained very much better negatives which have consistently indicated the monomolecular form over nearly fifty records.

<sup>5</sup> Harvey, E. N., *Am. J. Physiol.*, 1920, li, 580.

lated if desired. Thus from the decay curve which is tabulated in Table I and graphed in Fig. 1, we derive the absolute value of  $k$  as follows:

$$k = \frac{1}{t} \log \frac{A}{A - X}$$

If  $X$  becomes  $\frac{A}{2}$

Then

$$(3) \quad k = \frac{1}{t} \log 2$$

The time  $t$  needed to reduce the concentration  $A$  to half of its value is determined directly from the graph. For  $\log I$  at time 0, determined from the straight line intersection in Fig. 1, is 0.17.

Then

$$\begin{aligned} \log \frac{I}{2} &= \log I - \log 2 \\ &= \bar{1}.869 \end{aligned}$$

When  $I$  becomes  $\frac{I}{2}$ ,  $X$  becomes  $\frac{A}{2}$ , A reduction of  $\log I$  from

0.17 to  $\bar{1}.869$  corresponds to a time interval of 30.7 mm. of film, or 7.446 seconds of time. Using the usual time unit employed in calculating velocity constants, 1 minute, we find that, as 7.446 seconds is 0.124 minutes, then from equation (3)

$$\begin{aligned} k &= \frac{0.30103}{0.124} \\ &= 2.43 \end{aligned}$$

This gives the absolute value of the velocity constant if we are using Briggs' logarithms. The corresponding value of  $k$  for the natural system is:

$$\begin{aligned} k &= \frac{2.43}{0.4343} \\ &= 5.59 \end{aligned}$$

This absolute value of  $k$  will indicate the extraordinarily high speed at which this luminescent reaction is proceeding, even at room temperature.

*The Secondary Reaction.*—I have mentioned at various places the highly labile character of luciferin solutions which oxidize sponta-

neously in the absence of the enzyme, and without light production. It is not possible at present to state whether this spontaneous oxidation continues side by side with the catalytic oxidation when the enzyme has been added, or how important a part it plays if it be then present. In any case it proceeds at a much lower rate than the catalytic reaction, and does not affect the monomolecular reaction, and hence only affects the value of  $k$  in equation (2), without destroying the logarithmic form itself.

It is not possible, of course, to follow the kinetics of this secondary reaction in any direct way since it produces no light. I have attempted to ascertain by an indirect method whether the assumption of a monomolecular form for this reaction is correct. To three tubes containing originally identical quantities of luciferin solution (20 cc.) were added identical luciferase solutions (1 cc.) at successive times, the second addition being 10 minutes after the first, and the third addition 20 minutes later. All solutions were stirred from the instant of addition. The light from these tubes was allowed to fall for 90 seconds from identical distances upon three small neighboring areas of a strip of film. A calibration series was then impressed upon the record and the film developed. In 90 seconds over 99 per cent of the luciferin in such an aqueous solution has been oxidized with light production. It will later be seen that the total light given out when the reaction has gone to completion is probably a linear function of the initial concentration of luciferin. The decrease in total light therefore, in solutions which have stood until the secondary reaction has reduced the initial luciferin concentration, may be taken as a direct measure of such reduction. In one experiment I found that the total light emitted from the second tube, which had stood for 10 minutes, was 52.24 per cent of that emitted by its companion tube run previously. If the spontaneous reaction is monomolecular in character, the third tube run after 20 minutes should have shown a decrease in intensity to 27.29 per cent of the first tube. The actual total intensity observed in this tube was 28.45 per cent which agrees rather well with the expected value. Such data gives fair quantitative support to the hypothesis.

While this secondary or spontaneous reaction has entered in at various points to complicate the problem, it has also proven of value in one part of the study. It is greatly accelerated by a rise in tem-

perature, and I have used this characteristic to prepare luciferin solutions of different concentrations, as will shortly be described.

## 2. *Experimental Errors.*

In quantitative work with unstable organic substances, errors are certain to appear, no matter how carefully the manipulation may be

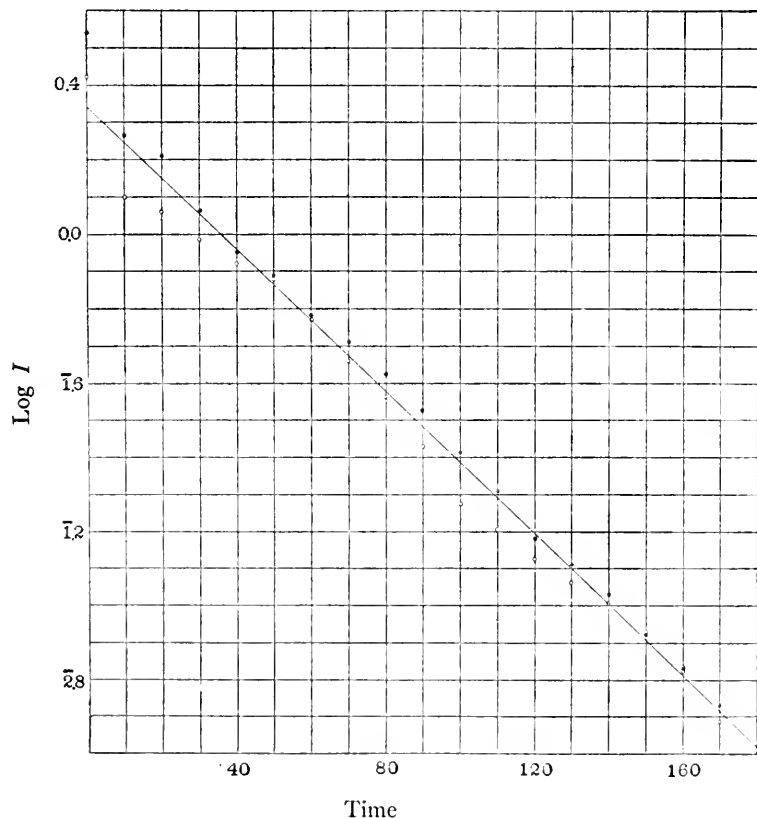


FIG. 2. Identical solutions. The values check within the photographic error. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

controlled. I have made every effort to hold all factors constant, except the one under investigation, but small and uncontrollable variations undoubtedly have been present. It is impossible, for instance to throw all of the enzyme solutions from their containing pipettes. A small amount remains upon the walls. This amount is

usually practically identical in each pipette but variations in enzyme strength of as much as 2 or 3 per cent may be produced in this way.

I have previously discussed the magnitude of the errors known to inhere in the photographic method itself. The question may well be raised, however, as to how closely it is possible to secure identity when simultaneous records are made using identical concentrations of both enzyme and substrate. I have plotted the data obtained in two such identical runs in Fig. 2. In practically all cases the differences between the two readings are within the error of the photographic method.

### 3. *The Influence of Stirring.*

I have previously mentioned that, in spite of the known character of the reaction, which is at least bimolecular, involving both luciferin

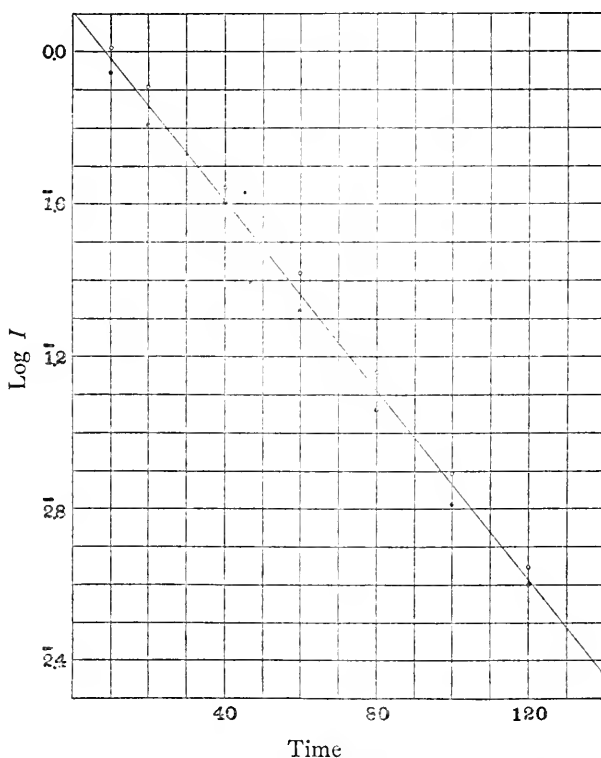


FIG. 3. Effect of stirring.  $\circ$  stirred;  $\bullet$  not stirred. Values check within the error of the method. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.



and oxygen, the decay curve follows the monomolecular form, because, under the conditions of the experiment, oxygen is always present in excess. The oxygen tension required for a maximum brightness of *Cypridina* luminescence is known to be quite small. Harvey<sup>5</sup> found that the brightness of luminescence in this animal is not affected by decreases in oxygen concentration until the tension has fallen below the value of 53 mm. of mercury. Above this value the oxygen may be considered to be in excess. It has not been possible in the present work to boil out solutions to such a low value of oxygen concentration, or to prevent its rediffusion during the pourings necessary in their manipulation.

I was interested to know, however, whether stirring would in any way affect the form of the decay curve. I found that stirring has no effect, and that two simultaneous records with identical solutions, one stirred, and the other not, follow each other side by side down the curve of decay, within the limits of the photographic error. The results of such an experiment are graphed in Fig. 3. Complete mixing of enzyme and substrate is evidently produced by the force with which the enzyme is added, and stirring does not add to its completeness.

#### 4. *The Influence of Enzyme Concentration.*

Actual concentrations of materials used are, of course, impossible to ascertain. Accurate relative concentrations of luciferase may, however, be obtained by dilution. Using this method I have studied the effect upon reaction velocity when a certain concentration of enzyme, which we will call  $C$ , is reduced by dilution to the values  $\frac{C}{2}$ ,  $\frac{C}{4}$ , and  $\frac{C}{8}$ . Both enzyme solutions are added simultaneously to identical luciferin solutions at the same temperature.

Typical records obtained in such a way are graphed in Fig. 4, in which the two enzyme concentrations were  $C$  and  $\frac{C}{2}$ . The slope of the straight line plotting gives at once the values of the velocity constants, as already shown in equation 2. The ratio of these slopes

therefore, represents the effect of the dilution upon the reaction velocity. In this case this ratio is 2.17, a slightly higher value than would be expected if reaction velocity were directly proportional to enzyme concentration. Such a variation might well arise from the experimental errors, but in the light of the data tabulated in Table

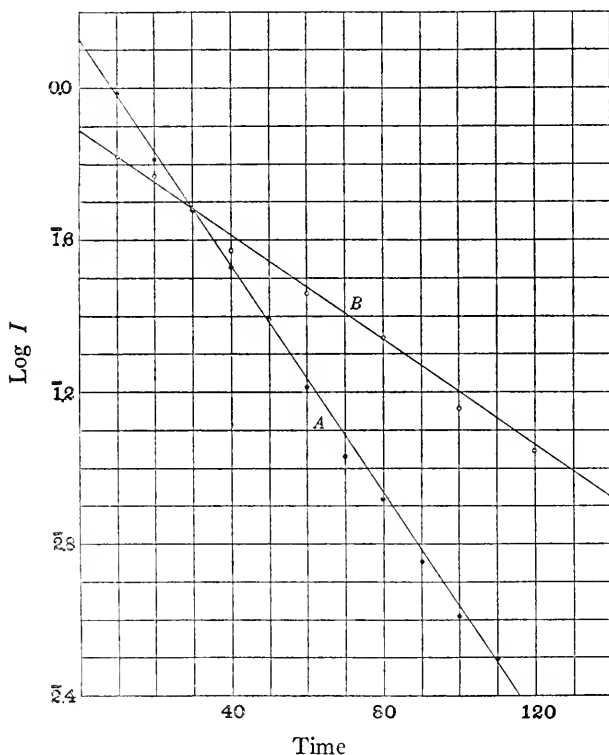


FIG. 4. Effect of enzyme concentration. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

$$k \text{ for } A \left( \frac{C}{2} \right) = 1.500$$

$$k \text{ for } B \left( \frac{C}{2} \right) = 0.690$$

$$\text{Ratio} = 2.17$$

III, it will be seen that the slope ratios are practically always (one exception) somewhat higher than the expectation for a direct proportionality. It can be stated that the velocity of reaction is very nearly proportional to enzyme concentration.

Upon the assumption of a strict proportionality the theoretical expectation would be that the initial light at zero time would be half as bright for  $\frac{C}{2}$  as for  $C$ . For from equation 2 if  $t = 0$ , then

$$\log I = \log Ak.$$

Now if  $k$  becomes  $\frac{k}{2}$ , as a strict proportionality would require,  $I$  must become  $\frac{I}{2}$ , since  $A$  is constant. The initial flashes, tabulated in Table III, are seen to be quite erratic, and not at all in line with this

TABLE III.

*Effect of Enzyme Concentration upon Reaction Velocity.*

Enzyme concentrations.	$k$	$\frac{k_1}{k_2}$	Initial light.		Initial flash	
			Calculated.	Ratios.	Observed.	Ratios.
$C$	1.660	2.16	3.020	1.82	Indeterminate.	
$\frac{C}{2}$	0.768		1.660		Indeterminate.	
$C$	1.168	2.38	1.079	1.92	1.778	1.41
$\frac{C}{2}$	0.490		0.5623		1.259	
$C$	1.500	2.17	1.340	1.71	3.311	2.04
$\frac{C}{2}$	0.690		0.7762		1.622	
$C$	1.064	3.94	1.905	3.09	3.162	2.80
$\frac{C}{4}$	0.270		0.6166		1.135	
$C$	1.007	4.93	1.480	2.32	3.357	2.79
$\frac{C}{4}$	0.204		0.639		1.202	
$C$	1.195	8.92	0.5598	3.84	0.8185	2.65
$\frac{C}{8}$	0.134		0.1456		0.3090	
$C$	1.356	9.54	1.393	5.58	2.291	5.56
$\frac{C}{8}$	0.140		0.2495		0.412	

theoretical expectation. Even the values calculated for the initial brightness from the intersections of the straight line plottings upon the zero time axis give poor approximation to the theoretical values. I believe that the presence of the initial flash is responsible in some way for this lack of coincidence, masking, as it does, the fundamental nature of the decay during the first second or two. I consider the very good approximation of the experimental values for reaction velocity with the expectation for a direct proportionality between it and enzyme concentration as being more significant and important than these initial light observations and calculations.

### 5. Influence of Luciferin Concentration.

Returning again to equation (2) we observe that if the value of  $A$ , or luciferin concentration, be reduced with all other factors held constant, the value of  $k$  is not affected by this reduction, and the slope of the straight line plotting, its graphical counterpart, is also unaffected. In other words, the theoretical expectation is that with two different luciferin concentrations the straight line forms should run parallel to each other.

In my first attempt to investigate this factor I adopted a dilution method similar to that used with success in reducing enzyme concentrations to known lower values. In diluting I first used distilled water, and then later, because this procedure diluted the yellowish pigments always present as well, I diluted with oxyluciferin solutions, boiled until complete oxidation of the original luciferin had been effected. For I found that the yellowish pigments gave a rather high extinction coefficient (about 0.3) for blue light which to the eye was a fair match for *Cypridina* light, and it at once became evident that while dilution with water would presumably not affect the shape of the decay curve, it would affect the magnitude of the light intensities observed all along it.

It appeared therefore that in order to obtain comparable values for two different luciferin concentrations it was necessary to control the pigment concentration. I did this by diluting with oxyluciferin solutions, whose pigment concentration was matched with that of the newly prepared luciferin solution which was to be studied, the two solutions being observed through a colorimeter until the concen-

tration of the oxyluciferin had been reduced by dilution to the proper value. I then prepared  $C$  and  $\frac{C}{2}$ ,  $C$  and  $\frac{C}{3}$ , and  $C$  and  $\frac{C}{4}$  luciferin solutions.

The study of such solutions gave erratic and somewhat curious results. In each separate curve the logarithmic relationship appeared quite definitely, but the expected parallelism in the straight line plottings did not develop. Instead, as Fig. 5 shows, the more dilute

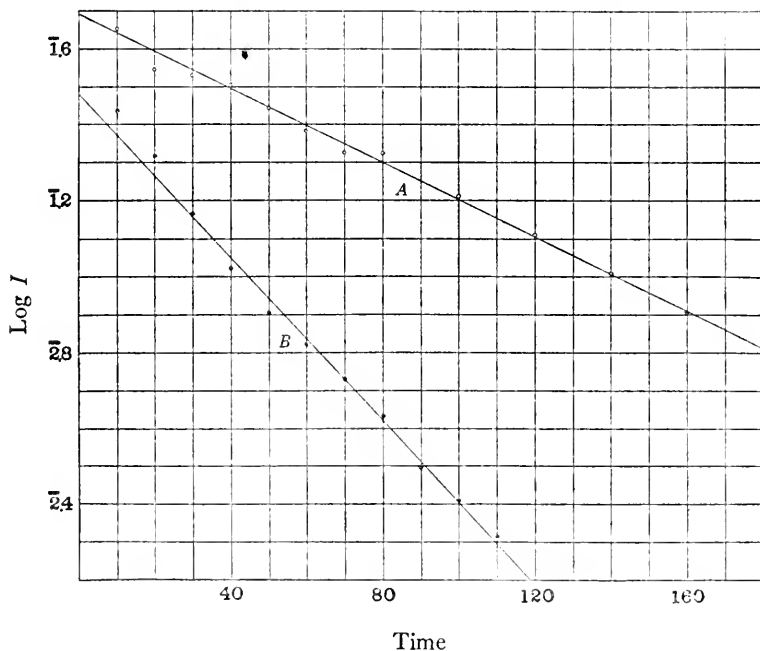


FIG. 5. Effect of dilution of luciferin solutions. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

$$k \text{ for } A (C) = 1.080$$

$$k \text{ for } B \left( \frac{C}{4} \right) = 0.484$$

$$\text{Ratio} = 2.23$$

solution appeared, in all but one case, to fall through the decay curve with a faster reaction velocity than its companion concentrated solution. Temperature was carefully controlled, and enzyme strengths were of course identical. I have tabulated some of these erratic results

in Table IV. By what must certainly be a chance coincidence the ratio  $\frac{k}{k_2}$  comes out very nearly the same for the three  $C$  and  $\frac{C}{2}$  records studied.

TABLE IV.

*Erratic Results Obtained with Dilution of Luciferin Solutions.*

Experiment.	Concentration.	$k$	$\frac{k_1}{k_2}$
4	$C$	0.570	1.442
	$\frac{C}{2}$	0.822	
33	$C$	0.845	1.444
	$\frac{C}{2}$	1.220	
34	$C$	0.778	1.401
	$\frac{C}{2}$	1.090	
35	$C$	1.060	1.374
	$\frac{C}{3}$	1.456	
32	$C$	0.704	1.506
	$\frac{C}{4}$	1.060	
36	$C$	0.484	2.23
	$\frac{C}{4}$	1.080	
37	$C$	0.910	0.831
	$\frac{C}{4}$	0.756	

I am not able to state what uncontrolled factor has been at work in these dilution experiments to produce this curious acceleration of velocity at lower concentrations of the luciferin. It may possibly be a difference in pH, or in salt concentration. In any case I have been able to determine that these results do not represent the real effect of changing luciferin concentration alone.

In my first attempt to study the possibility of obtaining identical decay curves with identical enzyme and luciferin solutions, I did not use the double pipette which has been described, but started the two reactions successively, the one 16 seconds after the other, so as to place the records side by side upon the film. I found that the resulting decay curves were not superposable, but in the straight line plotting ran parallel to each other. It seemed reasonable to suppose that in the intervening 16 seconds of time the spontaneous

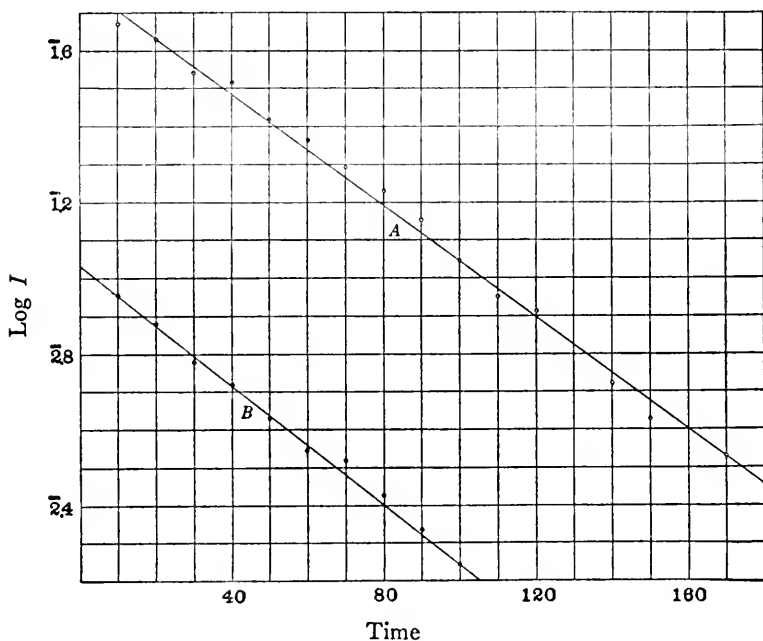


FIG. 6. True effect of different luciferin concentrations. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

$k$  for high concentration (A) = 0.737

$k$  for low concentration (B) = 0.790

oxidation in the second solution might have considerably reduced the luciferin concentration in that solution, with a change of the  $y$ -intercept of the straight line, but not of the slope, as above discussed. This experiment furnished the clue to a more satisfactory technique, which does not permit, it is true, of securing accurate previous infor-

mation as to relative luciferin concentrations, but does effect the production of differences in them with all other factors held constant.

The procedure adopted was the rather simple one of increasing the temperature of one sample of a luciferin solution, to hasten the secondary reaction, while its originally identical companion was held at a lower temperature. In the experiment shown in Fig. 6 one solution, giving curve *B*, was raised to a temperature of 55° for 3 minutes, and then cooled to the same temperature as its companion tube. Simultaneous records of the two solutions were then made. This method avoids any error due either to pigment changes or to pH fluctuations, or to any other unknown and uncontrolled factor which may have entered in to distort the previous results. The records thus obtained are so closely parallel as to admit of no other

TABLE V.

*Identity in Values for  $k$  Obtained with Luciferin Solutions in Which the Concentration in One Solution Has Been Diminished by the Secondary Reaction through Heating or Standing.*

Experiment No.	$k$ for high concentration.	$k$ for low concentration.
10	0.658	0.635
12	1.220	1.260
13	0.824	0.821
40	1.024	1.023
41	0.737	0.790

interpretation but that luciferin concentration does effect only the value of the  $y$ -intercept, and not the value of  $k$ , according to the theoretical expectation. I have tabulated the values of  $k$  for five such experiments in Table V.

While I have been unable to obtain such records from solutions in which the relative luciferin concentrations are accurately known in advance there is every reason to believe that, since the two plottings are parallel, in accord with expectation, the relative concentrations may also be arrived at from the data. For if  $A$  in equation (2) becomes  $\frac{A}{2}$ , then at zero time,  $I$  must become  $\frac{I}{2}$ , since  $k$  is constant; and if  $A$  becomes  $\frac{A}{4}$ , then  $I$  becomes  $\frac{I}{4}$ , and so on. So if, as in Fig. 6,



the logarithmic difference in the values of the two  $y$ -intercepts is 0.750, the initial brightness of solution *B* must have been 17.8 per cent of solution *A*, and the luciferin concentration must be represented by the same figure.

It follows that the total amount of light emitted from two solutions differing in luciferin concentration will be in direct proportion to those concentrations, for at every instant the light emitted by the one will be the same constant fraction of the light emitted by the other. I have previously used this relationship to determine indirectly the form of the reaction in the secondary or spontaneous reaction.

#### 6. The Temperature Coefficient.

I have made a brief preliminary study of the temperature coefficient, and upon the basis of the data which I have obtained can say that

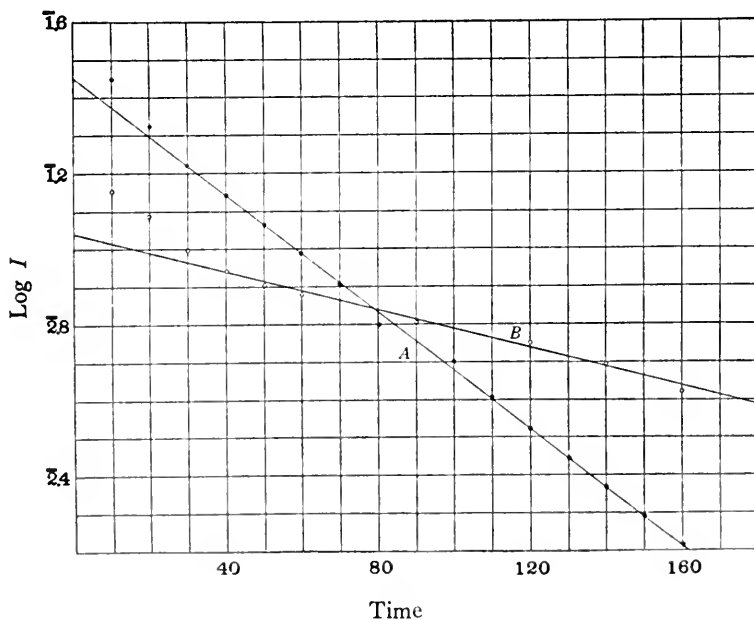


FIG. 7. The temperature coefficient. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

$$k \text{ for } A (24^{\circ}\text{C.}) = 0.775$$

$$k \text{ for } B (16.5^{\circ}\text{C.}) = 0.250$$

$$\text{Ratio} = 3.100$$

it is quite high for this reaction, the values being about 4.5 for the 15–25° interval, and 3.0 for the 25–35° interval. Fig. 7 will indicate the form of the records assumed in such a determination. From the data of this experiment the value of  $Q_{10}$  is calculated by Snyder's<sup>6</sup> formula

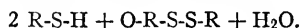
$$\left(\frac{k_1}{k_2}\right)^{\frac{10}{t_1-t_0}} = Q_{10}$$

Substitution of the observed values from the experiment in this equation gives  $Q_{10} = 4.625$  for the 15–25° interval. The actual value for 28–38° is found to be 2.851.

#### DISCUSSION.

The following out of the initial assumption of the work, namely that light intensity at any instant is directly proportional to the reaction velocity at that instant, has led to a fairly consistant picture of what occurs in the course of the bioluminescent reaction in *Cypridina*. The decay curve is seen to fulfill closely the theoretical expectation for a monomolecular reaction the velocity of reaction is found to be proportional to enzyme concentration, and the diminution of substrate concentration affects only, as it should, the value of the y-intercept, and not the slope of the straight line plotting. The very consistency of the results points strongly to the truth of the initial assumption, for which indeed there is a strong likelihood, on purely *a priori* considerations. I believe that the present observations will lend considerable quantitative support to the hypothesis of Trautz.

The monomolecular form of the decay curve indicates very clearly that we are dealing here with an oxidation process similar to the oxidation of leuco-methylene blue, as Harvey<sup>7</sup> has suggested, and not with a process of the type of the well known oxidation of cysteine to cystine by the dehydrogenation and union of two cysteine molecules. A reaction of the latter type may be written

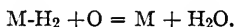


Presumably, with oxygen in excess, it would follow a bimolecular form. In the reaction under discussion, however, as in the oxidation

<sup>6</sup> Snyder, C. D., *Science*, 1911, xxxiv, 415.

<sup>7</sup> Harvey, E. N., *The nature of animal light*, New York, 1920, 128.

of leuco-methylene blue, a single molecule undergoes dehydrogenation according to the form



The present data are somewhat more difficult of interpretation when we turn to the conditions obtaining during the first few seconds of the reaction, for here we find a bright initial flash out of all accord with the succeeding values, and thereby masking the beginning of the reaction.

As far as I am aware, no report has ever been made, or perhaps has ever been possible, upon the reaction velocity of any organic catalysis during the first few seconds of its course. The reaction under consideration is unique in affording information as to reaction velocity at every instant along its course, and the photographic method which I have employed has given clear evidence concerning the rather striking character of the bright initial flash. The kinetics of no other enzymatic process that I know can be studied in any such direct way. In none other can the  $\frac{dx}{dt}$  of the reaction be immediately measured. It appears that this luminescent reaction may be thus peculiarly fitted for the making of further quantitative studies bearing upon enzyme theory.

I am convinced that the occurrence of the bright initial flash has a considerable theoretical significance. It has at times been observed, in the study of inorganic heterogeneous catalysis,<sup>8</sup> that there may occur high initial reaction velocities similar to that of the luminescent reaction in *Cypridina*. Such phenomena may be interpreted as due to the fact that at the very beginning of the reaction, the surfaces of the catalyst are clean, allowing a rapid adsorption of the substrates and a high reaction velocity. These initial conditions speedily vanish, for the collection of the resultants at the surfaces rapidly decreases the active masses, until an equilibrium is established between diffusing resultants, and new active material reaching the surfaces. The reaction then slows down, and straightens out into a consistent form which governs its later course.

<sup>8</sup> Unpublished work from the Department of Chemistry, Princeton University.

It has long been believed by many students of organic catalysis that enzymes and their associated substrates must really be heterogeneous systems, and many facts have pointed indirectly to the truth of this belief. In the present instance there can be no doubt that luciferase, the enzyme concerned, is a colloid, as are most enzymes (Harvey, 1920)<sup>9</sup> and a complex protein at that. There is every reason, therefore, for expecting heterogeneity.

Yet this expectation for enzyme reactions is rarely borne out by the result of kinetical studies. In such heterogeneous systems the rate of diffusion to the surfaces involved becomes an element in the situation, but this factor is usually completely masked by the relative rapidity of this diffusion as compared with the velocity of the reaction itself. Thus Arrhenius<sup>10</sup> states: "The study of the velocity of reactions in heterogeneous systems indicates that they behave very nearly in the same manner as homogeneous systems . . . . It depends on the circumstance that the diffusion goes on so rapidly that it does not perturb the chemical processes."

Even in a reaction which proceeds as swiftly as that under consideration, the diffusion rate must be even more rapid and therefore negligible, for the temperature coefficient is high, as usual for enzymatic processes. During the major part of the reaction, therefore, we must be measuring the rate of the oxidation process itself, and no hint of the heterogeneous nature of the system can be derived from the data. The initial flash is, however, in a different category, and I believe that it must be argued that the momentary appearance of this high reaction velocity when the enzyme is introduced is a direct indication of the heterogeneity of the system, to be interpreted in some similar way to that already stated for inorganic heterogeneous catalysis. The details of the mechanism can not yet be stated. It must be admitted that the surfaces of the enzyme particles can not be conceived to be initially completely clean, since, from the manner of their preparation, oxyluciferin is present. The major portion of the surfaces involved may still be free from such reaction products.

Under the experimental conditions of the present work it is certainly not possible to reach the high concentration values which must

<sup>9</sup> Harvey, E. N., *The nature of animal light*, New York, 1920, 141.

<sup>10</sup> Arrhenius, S., *Immunochemistry*, New York, 1907, 142.

exist for both enzyme and substrate in the bodies of luminescent forms. We have already seen that even *in vitro*, with comparatively dilute enzyme solutions, the velocity constant reaches a very high value. In those luminescent forms in which the mixing of luciferase and luciferin occurs within the body, concentrations must be much higher, and the reaction must be still further greatly accelerated, so that the light is confined practically to a single momentary flash. Even in *Cypridina* where the luminescent materials are ejected into the sea water, these must be very narrowly localized, and the velocity of reaction and the intensity of the light produced, must be far higher than we can secure in our laboratories. These bioluminescent reactions give a beautiful visual demonstration of the swiftness and efficiency of organic catalysis.

#### SUMMARY.

1. The decay curve of the light produced in the course of the luminescent reaction in *Cypridina* is, after the first second, in complete agreement with the theoretical expectation for a monomolecular reaction, if light intensity at any instant is assumed to be proportional to reaction velocity at that instant. It is shown that for such a reaction

$$\log I = -kt + \log Ak$$

and that the experimental values satisfy this equation.

2. The first second or two of the reaction is characterized by a brilliant initial flash, whose value is much too high to accord with the succeeding intensities and with the above formula. It is suggested that this initial high reaction velocity is an indication of a heterogeneous system.

3. Identical solutions run simultaneously give decay curves which check within the limits of the photographic error.

4. Stirring does not affect the reaction velocity or the form of the decay curve.

5. Reaction velocity is proportional to enzyme concentration, over the range of concentrations used in the study.

6. Changes in the concentration of the substrate do not affect the value of  $k$ , when all other factors are held constant. A diminution

of luciferin concentration results only in a decrease in the value of the  $y$ -intercept,  $\text{Log } Ak$ , the two straight line plottings for two different concentrations being parallel.

7. The temperature coefficient is high, being about 4.5 for the 15–25° interval, and 3.0 for the 25–35° interval.

In conclusion I wish to express my sincere thanks to Dr. E. Newton Harvey, of Princeton University, whose interest in this study has made its completion possible. I also wish to express my deep obligation for valuable assistance upon the physical aspects of the problem to Dr. Edward P. Hyde, Director of the Nela Research Laboratories, and to members of his staff, especially Dr. W. E. Forsythe, Dr. A. G. Worthing, Dr. E. Q. Adams, and Mr. M. Luckiesh, as well as to Dr. Charles Brush, who was kind enough to place a fellowship in the Nela Research Laboratories at my disposal for the summer months of 1921. The experimental methods were developed at that laboratory; the major part of the observations have been made at Princeton University.

# A NOTE ON THE ACTION OF CURARE, ATROPINE, AND NICOTINE ON THE INVERTEBRATE HEART.

By A. J. CARLSON.

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The observations recorded in this note were made in 1903-04 as an item in a more comprehensive work on the physiology of the invertebrate heart. These observations were not published, but some points were elaborated in greater detail on the *Limulus* heart, where unusual anatomical relations permit the analysis of the point of action of drugs, and this latter work on *Limulus* was reported in 1906 (Carlson, 1906-07). Having recently, with Dr. Luckhardt, become interested in the action of alkaloids on the nervous tissues in other automatic organs (lungs, gut, and arteries), the author was led to consult the earlier work on the invertebrate heart, and thus came across this item. Feeling that the observations are of some interest to general physiology they are now recorded as written up in 1904, except for the addition of two references (Carlson, 1906-07, 1909) in the bibliography.

The alkaloids used were of Merck manufacture. The species of invertebrates studied were: molluscs (*Octopus*, *Loligo*, *Ommastrephes*, *Mytilus*, *Mya*, *Tapes*, *Platydora*, *Venus*, *Pecten*, *Cryptochiton*, *Lucapina*, *Haliotis*, *Natica*, *Sycotypus*, *Aplysia*, *Bulla*, *Pleurobranchæa*, *Montreina*, *Triopha*, *Limax*, *Ariolimax*, and *Helix*), and arthropods (*Palinurus*, *Cancer*, and *Limulus*).

## 1. The Action of Curare, Atropine, and Nicotine on Central and Peripheral Ganglia.

The action of alkaloids in the invertebrates has been the subject of numerous studies. The older researches are cited and reviewed in

\*I am indebted to the directors of these laboratories for the facilities so liberally extended.

the works of Plateau (1880) and Krukenberg (1880). According to Bert (1867) and Yung (1879, 1881), the action of curare in the crustaceans and in the cephalopod molluscs is the same as in the vertebrates, only less strong. In the lamellibranch molluscs Yung did not obtain a permanent paralysis by curare. Vulpian (1879) states that curare paralyzes both arthropods and molluscs, but its action is not as strong as in the vertebrates. Krukenberg holds that the action of curare in crustaceans and molluscs is mainly on the central nervous system, but there is also paralysis of motor nerve endings. Biedermann (1890) and Fürst (1890) states that curare will paralyze the motor nerve endings in *Lumbricus* but this is denied by Straub (1900).

A drug may cause paralysis by depressing the motor nerve endings or by paralyzing the central nervous system. My own results go to show that the primary action of curare and nicotine in arthropods and molluscs is on the central nervous system and the peripheral ganglia and not on the motor nerve endings in the muscle. The action on the nerve centers is a primary stimulation followed by temporary or permanent paralysis if the dose is of sufficient strength.

There are great differences in the tolerance or degree of resistance to the action of curare in the different invertebrates. The nudibranchs and the pulmonates (excepting *Ariolimax*) are very sensitive to the drug. *Octopus* is far more resistant than the decapods, and of the latter *Ommastrephes* is more resistant than *Loligo*. Of the arthropods, *Limulus* shows the greatest resistance to the drug.

The stimulating action of curare on the nerve centers appears immediately on the application of the solution. In the squid the stimulation results in spasms and tetanus. The primary stimulation is less in evidence in the crustaceans. In the gasteropods it appears in prolonged and extreme contraction of the body muscles. In all the animals studied stimulation of the motor nerves causes contraction of the skeletal and visceral muscles after a dose of curare that completely paralyzed the central nervous system.

Atropine appears to paralyze motor nerve endings to some smooth muscles (*e.g.* lungs, gastrointestinal tract) in vertebrates. The body muscles of arthropods are of the transversely striated type, but the muscle of molluscs approaches more closely to the smooth variety.



My experiments on several classes of molluscs with the view of paralyzing the motor nerve endings in the muscle by atropine have yielded uniformly negative results. So far, then, we know of no drug that will paralyze the motor nerve endings in invertebrates without materially depressing the muscle itself, after previous paralysis of the central ganglia.

## 2. *The Action of Curare, Atropine, and Nicotine on the Heart Rhythm.*

A review of the literature on the action of the alkaloids on the invertebrate heart discloses considerable disagreement between the results of different observers. Ransom (1884) found that curare in sufficient concentration accelerates the heart rhythm of *Octopus* and *Helix*. According to Yung (1881), curare has sometimes a depressor and sometimes a stimulating action on the heart of lamelli-branches. Plateau (1880) states that curare has a depressor action on the crustacean heart, and according to Dogiel (1877) the drug has no action whatever on the heart of the *Corethra* larva.

Nicotine accelerates the heart rhythm both in crustaceans and in molluscs (Yung, 1881, Plateau, 1880).

Plateau states that atropine has a depressor action on the crustacean heart. Yung and Ransom found that it acts as a stimulant on the molluscan heart. Dogiel states that atropine of sufficient concentration to affect the heart of the *Corethra* larva at all has a depressor action. For an account of the action of these alkaloids on the tunicate heart the reader is referred to the papers by Schultz (1901) and Hunter (1903). My own work does not include the heart of tunicates.

In order to study the action of these drugs on the heart it is, in the first place, necessary to sever the connection of the heart with the central nervous system. All the animals worked on are provided either with inhibitory or accelerator cardiac nerves or both (Carlson, 1909). The action of the alkaloids on the heart when introduced into the intact animal is therefore complicated by their action on the central ganglia or brain and on the peripheral ganglia other than those in the heart. Satisfactory results can for that reason be obtained only on the denervated or excised heart. The solutions of the alkaloids may be applied to the surface of the excised heart and empty heart,

or the heart may be filled with the solution through a cannula. Both these methods were used.

It is furthermore necessary that the solvent is neutral or almost neutral to the heart. Distilled water applied to the molluscan and crustacean heart accelerates the rhythm and produces tonus contractions. It will therefore not do to use distilled water as the solvent. Sea water is almost neutral to the heart of all the marine animals worked on. It has a slight stimulating action but this appears very gradually and only after long immersion of the heart in the sea water. The solution of the alkaloids in sea water will in consequence give fairly accurate results. A still better solvent is the blood plasma of the animals themselves, and this was used in nearly all cases. I was not able to obtain an artificial salt solution that proved to be neutral to the heart of the pulmonates. A solution of the drugs in the blood plasma was the only method available in the work on the snail and the slug heart.

The graphic method was used for recording the change in the heart rhythm.

*Solutions of Curare, Atropine, and Nicotine of Sufficient Concentration to Affect Appreciably the Heart Have a Primary Stimulating Action.*—The acceleration of the rhythm is followed by depression and, if the concentration of the alkaloids is great, by complete cessation of the rhythm, the heart remaining excitable to direct stimulation. Nicotine is in every case the strongest stimulant. There does not seem to be any great difference between the stimulating action of atropine and that of curare.

The hearts of the various invertebrates studied differ greatly in their sensitiveness to the action of the drugs. In the weakest concentrations of curare the stimulating action appears in augmentation of the rate and strength of the beats, and by gradually increasing the concentration this augmentation passes into a condition of incomplete tetanus. This tetanus may be maintained for 5 to 8 minutes, especially in the heart of lamellibranchs and gasteropods and in the gill ventricles of the squid. The relaxation is gradual and may be accompanied by a feeble rhythm. After the rhythm has been abolished by the action of a strong (1 per cent) solution of curare it can usually be restored by bathing the hearts in plasma. This is not

true for the heart of the crab. A solution of curare strong enough to produce incomplete tetanus of the heart in this species abolishes the rhythm permanently.

The latent period of the stimulating action of curare varies with the sensitiveness of the particular animal to the drug as well as with the mode of application of the solution to the heart. In the molluscs the alkaloid acts more quickly when poured into the cavity of the heart than when applied to the surface of the heart. This is probably due to a difference in permeability. When the solution is applied to the surface of the heart it has to penetrate the epicardium in order to act on the nervous and the muscular tissues, while there is no endothelium lining the heart cavity. In the crustaceans the drug acts equally quickly whether applied to the surface of the heart or introduced into the heart cavity. When the curare solution is applied to the nerve cord on the dorsal side of the heart of *Limulus* the action is practically instantaneous. The crustacean heart is more sensitive to curare than the molluscan heart (with the exception of the heart of *Loligo*). Between closely related molluscs there may be a great difference in sensitiveness. Thus the ventricle of *Ariolimax* continues to beat with an accelerated rhythm for 25 to 30 minutes in a 0.5 per cent solution of curare, a concentration producing cardiac tetanus within a minute in the ventricles of *Helix* or *Limax*.

The primary stimulating action of atropine and nicotine on the heart is to all appearances very similar to that of curare. Nicotine has to be used in dilutions of 1:1,000 to 1:10,000 to exhibit the true stimulating action. In greater concentrations it usually stops the arthropod heart at once without any primary stimulation. A strong solution of curare has sometimes the same effect on the feebly pulsating crab heart. This difference is probably due to differences in permeability.

The strong stimulating action of curare in the invertebrate heart (without exception) is in contrast to the relatively slight action of this drug on the heart of vertebrates. Atropine and nicotine, on the other hand, are strong stimulants also to the vertebrate heart. The stimulating action of the curare solution may in part be due to the action of potassium salts which are present as impurities in commercial curare. I have found that a slight concentration of the potassium chloride in

the blood of *Limulus* has a powerful stimulating action on the ganglion cells in the heart. The solution of these drugs in the isotonic solvents changes the osmotic pressure of these liquids and a slight change in the osmotic pressure of the blood has itself an effect on the heart.

The point of the action of these alkaloids in the heart is not yet known. On the myogenic theory of the heart beat their stimulating effects may be due to action on the accelerator nervous mechanism or to a direct action on the heart muscle. On the neurogenic theory the augmentation of the rate of the beats can hardly be accounted for except by direct action on the local ganglia, while the increased amplitude of the contractions may be due to action directly on the muscle. To answer the question whether these and other alkaloids act on the nervous or on the muscular tissue in the heart or on both, several investigators have studied their action on the embryonic heart on the theory that the heart on the embryo begins to beat before any nervous elements are present. Pickering (1893, 1894-95) found that atropine and nicotine accelerate the embryonic (chick) heart, while strong solutions of atropine depress the rhythm without any primary stimulation. Cyrillo (1901) states that atropine depresses the embryonic heart. This investigator finds, moreover, that the action of the principal alkaloids on the embryonic heart is the same as on the heart of adults, from which he concludes that these drugs act primarily on the heart muscle, their action on the nervous tissue in the heart being entirely of a secondary character.

I have come to the very opposite conclusion, or that the primary action of the alkaloids is on the ganglion cells in the heart and not on the muscle. This conclusion is based on the results on the heart of *Limulus* (Carlson, 1906-07). For this line of study the *Limulus* heart is prepared in the following manner:

The nerve cord on the dorsal side of the heart is extirpated in the first three segments, which leaves this part of the heart free from ganglion cells, the rhythm being maintained by the impulses reaching the muscle from the nerve cord of the middle third of the heart along the lateral nerves. It is desirable to isolate further the ganglionated and the ganglion-free ends of the heart by excision of the heart muscle for a distance of 1 cm. in the third segment. This dissection can be

done without the least injury to the lateral nerves. This makes an ideal preparation. It is a simple matter to apply a solution to the first two segments and absolutely prevent it from getting to the hind portion of the heart and, conversely, to bathe the nerve cord on the posterior end of the heart in a solution without the solution reaching to the first two segments. Any change in the rhythm of the first two segments on application of a drug to the nerve cord of the posterior end of this heart preparation can be due only to a change in the activity of the ganglion cells which maintain the rhythm. And again, the change in the rhythm of the first two segments produced by a solution applied to these segments alone is evidently not an action on ganglion cells but must be an action on the nerves and nerve endings in the muscle or on the muscle itself if we assume that local reflexes play no rôle. For accurate determinations of the changes in the rhythm in the two anterior segments in this preparation, the graphic method was always used.

On this preparation an extended series of tests of the principal alkaloids were made, the results yielding the conclusion expressed above. All the alkaloids tested act on the nerve cord. Some of them, especially veratrin, digitalin, and nicotine act also on the muscle, and it is probable that all of them act on the muscle if in very strong solution; but their action on the nerve cord or ganglion cells is much more rapid and intense, and all the alkaloids, moreover, act on the nerve cord in a dilution which has no or at least a very slight and gradual action on the muscle. Thus a solution of 1 per cent curare or atropine in plasma or sea water stimulates the nerve cord powerfully at the very instant of application, while no change in the rhythm follows its application to the muscle. Further work in this line will probably show that difference in the action of the alkaloids on the nervous and on the muscular tissues in the heart is only one of degree, the ganglion cells being more permeable to the drugs and the muscle cells having lower excitability.

The action of these alkaloids (curare, atropine, and nicotine) on the whole heart of crustaceans and the molluscs is in all essentials the same as their action on the nerve cord or ganglion cells in the *Limulus* heart. The conclusion seems obvious that their action on the crustacean and molluscan heart is also primarily on the ganglion cells.

### 3. *The Effects of Curare, Atropine, and Nicotine on the Cardioresgulative Nervous Mechanism.*

Curare, atropine, and nicotine do not paralyze the cardioaccelerator nerves in the gasteropod molluscs or the intrinsic motor nerves in the heart of *Limulus*. The following experiment on *Ariolimax* may be quoted as typical.

Apr. 4, 1904. Heart of *Ariolimax*.

9.35 a.m. Heart exposed; stimulation of visceral nerve effective. Heart bathed in a 0.5 per cent curare solution in plasma; acceleration.

9.45 a.m. Stimulation of visceral nerve effective. Curare continued.

10 a.m. Stimulation of visceral nerve effective. Curare continued.

10.25 a.m. Stimulation of visceral nerve effective. Curare continued.

10.45 a.m. Accelerator action of nerve partly impaired. Curare solution replaced by blood.

11.10 a.m. Stimulation of visceral nerve accelerates heart.

11.30 a.m. Stimulation of visceral nerve accelerates heart. Blood replaced by 0.5 per cent curare solution. Acceleration of rhythm.

11.50 a.m. Stimulation of visceral nerve has only a very slight action on heart. Rhythm very feeble and slow.

12.15 p.m. Stimulation of visceral nerve still accelerates heart. Curare solution replaced by blood.

1.30 p.m. Heart quiescent. Stimulation of visceral nerve (interrupted current) produced a series of beats in ventricle.

The diminution of the influence of the motor nerves on the heart after prolonged action of these drugs is evidently due to diminished excitability of the heart. The final failure of the accelerator nerves appears only after such a prolonged action of the alkaloids on the heart that the rhythm is almost, if not entirely, abolished. In *Ariolimax* it is possible to produce a series of contractions in the ventricle brought to a standstill by curare, atropine, or nicotine on stimulation of the visceral nerve with the tetanizing current.

I am not in a position to say whether these alkaloids paralyze the accelerator nerves that connect the heart of *Limulus* with the central nervous system, but neither curare, atropine, nor nicotine abolishes the action of the nerve fibers that pass from the nerve cord on the dorsal side of the heart to the heart muscle.

*Continued Action of Solutions of Curare, Atropine, or Nicotine on the Heart Abolishes the Influence of the Inhibitory Nerves on the Heart*

*both in the Molluscs and the Arthropods.*—The paralysis of the inhibitory nerves may be only temporary, their function being restored by bathing the heart in blood. The paralysis is not produced except by a concentration of the drugs that causes marked primary acceleration of the heart rhythm. I did not find any essential difference in the paralyzing action of the three alkaloids.

In the lamellibranchs (*Tapes*, *Venus*) the curare solution may act for 20 to 30 minutes before the action of the inhibitory nerves on the heart is abolished. In *Helix* and *Limax* the action is much quicker, or in 4 to 8 minutes. The action is quicker the stronger the solutions. In *Loligo* the injection of a few drops of 0.5 per cent curare or atropine, or 0.1 per cent nicotine in the cephalic vena cava paralyzes the cardio-inhibitory nerves in 4 to 5 minutes. I have not succeeded in restoring the function of the nerves in *Loligo* after this paralysis. In *Limulus* the concentration of the drugs must be sufficient to cause great augmentation of the rhythm in order to abolish the influence of the inhibitory nerves on the heart, in which case the paralysis is brought about in a few minutes. In weaker solutions it requires much longer time and may in fact not occur at all.

At what point do these alkaloids act to produce the paralysis of the cardioinhibitory nerves? Atropine paralyzes the vagus fibers in the vertebrate heart, and it is generally held that this action is on the inhibitory nerve endings in the muscle. Nicotine, on the other hand, is supposed to paralyze the synapses of the vagus fibers with the inhibitory ganglion cells in the heart. Turning now to the heart of *Limulus*, it is certain that atropine as well as curare and nicotine act on the ganglion in order to paralyze the inhibitory nerves. If the action of the drugs is confined to the heart muscle and the nerves and the nerve endings in the muscle no paralyzing effects are produced. It would therefore seem that the action of these alkaloids, in abolishing the action of the inhibitory nerves on the heart, is on the ganglion cells in the heart or on the endings of the inhibitory nerves in connection with these cells.

While it is certain that curare, atropine, and nicotine in sufficient concentrations paralyze the cardioinhibitory nervous mechanism in molluscs and arthropods, this action requires, on the whole, a greater concentration of the drugs than that sufficient for the paralysis of the cardiac vagi in the vertebrates.

## SUMMARY.

1. The alkaloids (curare, atropine, and nicotine) in molluscs and arthropods stimulate and paralyze the central nervous system and peripheral (visceral) ganglia, but do not paralyze the motor nerve endings to skeletal or visceral muscle.
2. They stimulate and paralyze the denervated heart.
3. They paralyze or block the cardioinhibitory nerves, but not the cardioaccelerator nerves.
4. In the *Limulus* heart these drugs act primarily on the heart ganglion, not on the heart muscle or the intrinsic motor nerve fibers.

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# THE EFFECT OF TEMPERATURE ON THE PHOTOTROPIC RESPONSE OF NECTURUS.

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It is a well known fact that *Necturus maculosus* is negatively phototropic. According to Eycleshymer (1906, 1908, and 1914) Reese (1906), and Pearse (1910), the animal always avoids sunlight in its natural environment as well as in the laboratory. They conclude that the skin is the important receptor for the photic stimulus since eyeless animals behave similarly to normal ones. It is also known that *Necturus* is sensitive to temperatures below 5° and above 25°C. (Reese, 1906; Sayle, 1916). I have found that the animals become more active when transferred from a temperature of 20° to one of 2°C., but that after several hours of exposure to the low temperature, they become very sluggish. As the temperature increases they show increased activity until death ensues between 35° and 40°C. Violent spasmodic movements are characteristic of such high temperatures previous to death.

When illuminated, therefore, it may be assumed that the reaction time of *Necturus* will vary according to the intensities of the thermal and photic stimuli. The experiments reported here were performed to determine the effect on the reaction time of variations of the temperature in normal and eyeless animals, and to express it in quantitative form.

The animals were kept in a large aquarium with running water at 20°C. They were thus adapted to light of very low intensity and to a medium temperature. The animals were tested singly in a dark room, where the temperature and the light intensity could be easily controlled. They were placed in a rectangular blackened dish (170 cm. long, 11 cm. wide, and 8 cm. deep), and allowed 15 minutes for acclimatization. At the beginning of each trial, the animal was oriented in the center of the dish, with the longitudinal axes of the animal

and the dish parallel. One of the Mazda glowers was fixed above, so that the beam of light (10 cm. in diameter) centered on the head. When the glower was turned on, the animal crawled posteriorly until the head was out of the beam of light. This interval was designated the reaction time, and was measured by a stop-watch to the nearest second. The light was then turned out, and the animal given a rest period of 1 minute before the next trial began. Four intensities of light were used at four different temperatures on six animals. Ten trials were sufficient to obtain accurate averages before fatigue became apparent.

It was soon discovered that the reaction time of *Necturus* does not change much with the variations of light intensity from 1,875 candle meters to 8,000 candle meters. At a constant temperature of 22°C. and illumination of 1,875 candle meters, the average of sixty trials (ten on each animal) was 2.8 seconds while at 8,000 candle meters it was 3.1 seconds. At 2°C. the average for 1,875 candle meters was 8.1 seconds, and for 8,000 candle meters it was 8.3 seconds. The reaction time was not analyzed as to its exposure and latent periods, which were very short. Crawling began almost immediately after the light was turned on, so that the reaction time is really a measure of the time necessary for the animal to crawl out of the lighted area. Since it is improbable that this constitutes an exception to the Bunsen-Roscoe law, it is evident that in all the trials the maximum amount of stimulating substance was produced in a very short time, even at the lowest intensity. This would be true if the velocity of the photochemical reaction is high enough at 1,875 candle meters. The determination of the exposure period in its relation to the photic intensity was reserved for future investigation. In all the temperature tests only one intensity of light was used, 4,200 candle meters.

From Fig. 1 it will be seen that the reaction time varies inversely to the temperature. Between 2° and 32°C. the temperature coefficient gradually diminishes, as it does in other animals (Hecht, 1919), although its numerical value is not especially significant, since the several chemical processes underlying the reaction time of *Necturus* have not been analyzed. At 32°C. the animal probably moves as fast as its anatomy will allow, without showing the spasmic movements characteristic of temperatures above 35°C. The curve expresses in

definite form the effect of temperature on the phototropic response of *Necturus*, and it may be concluded that the decrease in the reaction time is due to an increase in the velocities of the chemical processes

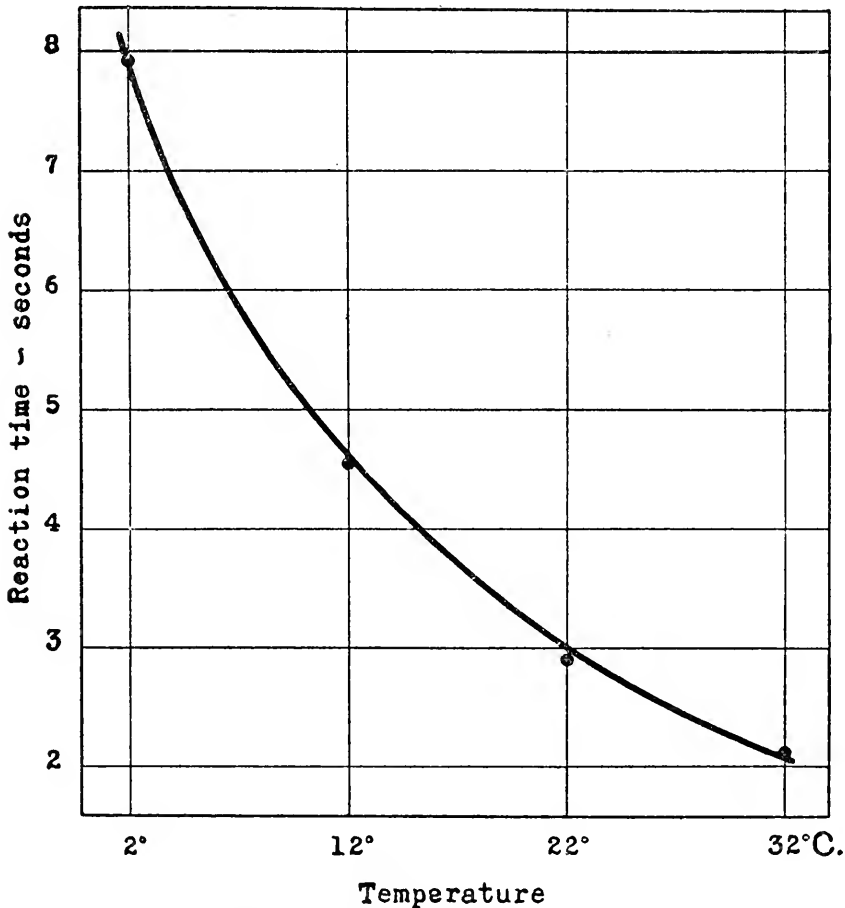


FIG. 1. Relation between temperature and reaction time. Each point is the average of sixty trials; ten on each of six animals. Intensity of light, 4,200 candle meters.

which cause muscular movements, and not to any effect of the temperature upon the photochemical reaction.

48 hours after the eyes had been removed, the six animals were tested again at each of the four temperatures, and with a light inten-

sity of 4,200 candle meters. The averages obtained were almost identical with those from normal animals. It is therefore concluded that the skin is the important receptor for the photic stimulus.

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# EFFECT OF COCAINE ON THE GROWTH OF LUPINUS ALBUS. A CONTRIBUTION TO THE COMPARATIVE PHARMACOLOGY OF ANIMAL AND PLANT PROTOPLASM.

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## INTRODUCTION.

The effects of chemicals on plants have in some respects been studied very thoroughly and in other respects almost not at all. Those drugs or chemicals which have been intensively investigated in this connection are the various salts or more accurately speaking ions which are necessary for the nutrition of various plants. The vast amount of work in plant physiology which has been done on this subject of plant nutrition and plant metabolism has been probably more thorough than the analogous observations on animals. At any rate the plant physiologist is able to produce a more perfect nutritive solution for the growth of plants than the animal physiologist can for the study of animal organs or tissues. While this phase of plant chemistry has received all the attention demanded by its importance, the influence of other chemicals or drugs and poisons on plant life has been barely touched upon. Yet the few contributions along these lines which have appeared, emphasize its importance. If we define pharmacology<sup>1</sup> as some authors do, as “. . . the study of the changes induced in living organisms by the administration in a state of minute division of such substances as do not act merely as foods” then the field of what we might term *phyto-pharmacology* is virgin soil. The effects of drugs or poisons on plants have been very little studied. The drugs which have perhaps received slightly more attention in this

<sup>1</sup> Cushny, A. R., A textbook of pharmacology and therapeutics or the action of drugs in health and disease, Philadelphia and New York, 4th edition, 1906.

respect are metallic salts. The effects of these on sprouting and growth have been studied by some authors such as Brenchley (1), Bokorny (2), and a few others. The deleterious effects of some gases have also been touched upon by some authors, such as Crocker and Knight (3), Crocker, Knight, and Rose (4), and others, and the effects of ether on flowers have been investigated by other plant physiologists (5). The influence of that most important class of drugs or poisons known as the alkaloids on the growth of plants, has, however, with the possible exception of nicotine (6) practically been left untouched.

In connection with the comparative study of the effects of cocaine and its decomposition products on various animal organs and tissues carried on by one of the authors, it was thought of interest to inquire into the effects of the same compounds on living organisms belonging to the plant kingdom and an investigation on the subject was accordingly undertaken with the kind cooperation of Professor Livingston of the Laboratory of Plant Physiology of this University. The results obtained were so interesting in themselves, and even more so as compared with the data obtained from animal work, that they are deemed worthy of publication.

#### *Methods.*

In the present investigation the effect on the growth of cocaine and its decomposition products were studied, in terms of elongation, of the roots of the seedlings of *Lupinus albus*. This lupine can be very easily germinated and the single straight root of the seedling can be readily measured. The procedure employed in the present experiments was as follows. The dry seeds were soaked overnight in tap water at ordinary temperature. On the following day the swollen seeds were planted with the hilum downward in moist, finely ground sphagnum moss. The planted seeds are placed in a thermostat and left at a constant temperature of 20°. On the third day after planting, the roots of the seedlings are of convenient length for measurement and are ready for study. After recording the exact length of a root it is placed in an upright test-tube of hard glass containing nutrient solution, the seed resting on the upper edge of the tube. The solution employed was the so called Shive solution

which contains calcium nitrate, magnesium sulfate, and monopotassium acid phosphate (7). Such a solution was prepared by mixing 10.4 cc. of 0.5 molar solution of calcium nitrate, 30 cc. of 0.5 molar solution of magnesium sulfate, and 36 cc. of 0.5 molar phosphate, with distilled water sufficient to make 1 liter. The normal growth of the lupine rootlets was studied by immersing the seedlings in a mixture of normal Shive solution with an equal part of distilled water. The effect of cocaine and other drugs was studied by dissolving chemicals in distilled water and mixing such drug solutions with equal parts of the normal Shive solution. After measuring accurately the length of each root and placing the seedlings in the control and drug solutions, the whole was again put in the incubator and left at a constant temperature of 20°C., and the effect of various chemicals on the growth of the roots was determined on the following day; that is, at the end of 24 hours. Ten seedlings were placed in the control solution, of one-half Shive solution and one-half water, and ten seedlings were employed for the study of each drug solution. In most of the experiments the volume content of the test-tubes used was about 10 cc. In the case of a few rare and valuable chemicals test-tubes of shorter length, holding about 5 cc., were occasionally employed.

The influence of the following substances, on the growth of lupine roots was investigated: cocaine hydrochloride, sodium benzoate, methyl alcohol, methyl benzoate, ecgonine hydrochloride, benzoyl ecgonine, and various mixtures of these drugs. It is well known that the cocaine molecule can be easily decomposed by hydrolysis, yielding *ecgonine*, *methyl alcohol*, and *benzoic acid*. The anesthetic properties of cocaine in animals are dependent on the chemical union of these three components. A simple mixture of the three will not give the same pharmacological effects as the chemical combination in the form of the cocaine molecule. This is true not only in regard to the anesthetic properties of cocaine but, as previously shown by one of the authors with various collaborators, also holds good in regard to the action of cocaine on the central nervous system (8), on skeletal muscle (9), and on smooth muscle. It was for this reason that it was deemed desirable to inquire into the effect on the lupine root, not only of cocaine itself but also of its various decomposition products.

All of the above substances were studied in various concentrations, the object being to ascertain, in the first place, whether they were toxic to the roots at all and if so to determine their limits of toxicity; that is, the minimal concentrations of the drug which produce a retardation in growth or other toxic effects.

#### EXPERIMENTAL.

*Effect of Cocaine.*—Cocaine hydrochloride was employed in the experiments, the chemically pure salt was dissolved in distilled water,

TABLE I.

*Relation between Concentration of Cocaine Hydrochloride and Growth of Lupine Roots.*

Concentration of cocaine hydrochloride.		Growth increment, for 24 hrs., expressed as percentage of corresponding increment in control test.
<i>mol per liter</i>	<i>per cent</i>	
0.2, 0.1, 0.08, 0.06	6.8, 3.4, 2.7, 2.0	No growth.
0.05	1.7	64
0.01	0.34	89
0.005	0.17	100
0.001	0.03	100
0.0005	0.17	100
0.0001	0.003	119, 93
0.00005	0.0017	80, 95
0.00001	0.0003	82, 121
0.000005	0.00017	91, 138
0.0000005	0.000017	90, 116
0.0000001	0.000003	100

and various concentrations of the solution were mixed with equal parts of normal Shive solution, the plants being immersed in the mixture. The results of the experiments are shown in Table I. In the first column are indicated the concentrations of the cocaine hydrochloride expressed as molar, and in the second column are given the equivalents of these concentrations in terms of per cent by weight. In the third column the mean growth increment of the seedlings in the cocaine solutions is indicated as compared with the corresponding normal or control increment, for 24 hours. It will be seen that growth of these roots is affected only by rather strong solutions of cocaine.



The minimal concentration required to inhibit growth completely was 0.06 molar, or about 2 per cent. Solutions slightly more dilute (e.g. 0.05 molar, or 1 per cent, allowed a growth increment of 64 per cent), and still weaker solutions did not impair the growth at all. In some of the experiments, indeed, as for instance in concentrations of 0.000005 molar and 0.0001 molar, there was even a suggestion of a stimulation in growth. This was not, however, definitely established, because of lack of cocaine. The significance of the toxic dose of cocaine for the lupine as compared with that of the same drug for animal tissues will be discussed below.

*Toxicity of Ecgonine and Benzoyl Ecgonine.*—Cocaine on being hydrolyzed readily yields the base ecgonine. Neither ecgonine nor

TABLE II.

*Relation between Concentration of Ecgonine Hydrochloride and Growth of Lupine Roots.*

Concentration of ecgonine hydrochloride.		Growth increment, for 24 hrs., expressed as percentage of corresponding increment in control test.
<i>mol per liter</i>	<i>per cent</i>	
0.005, 0.0025	0.11, 0.055	No growth.
0.001	0.022	78
0.0001	0.0022	83
0.00005	0.0011	85
0.00001	0.00022	100
0.000005	0.00011	100

benzoyl ecgonine exhibit the characteristic pharmacological properties of cocaine in animals. The effects of these compounds on the growth of lupine are shown in Tables II and III. Table II gives the effects of various concentrations of ecgonine hydrochloride, while Table III exhibits the results of experiments with benzoyl ecgonine. It will be noted that the minimal concentration of ecgonine hydrochloride which produced complete inhibition of growth was 0.0025 molar, or 0.055 per cent. Weaker dilutions ranging from 0.001 to 0.00005 molar, inclusive, permitted growth, though considerably retarding it. In concentrations of 0.00001 molar no deleterious effect on the growth of the roots was evident. It is thus seen that ecgonine is more toxic to lupine roots than is cocaine itself.

An examination of Table III reveals the fact that the toxic dose of benzoyl ecgonine is larger than that of ecgonine itself; in other words the benzoyl ecgonine is less toxic than ecgonine. In order to inhibit growth completely, concentrations of 0.1 molar, or 3.29 per cent, were required. A concentration of 0.1 molar, and lower ones, did not retard

TABLE III.

*Relation between Concentration of Benzoyl Ecgonine and Growth of Lupine Roots.*

Concentration of benzoyl ecgonine.		Growth increment, for 24 hrs. expressed as percentage of corresponding increment in control test.
<i>mol per liter</i>	<i>per cent</i>	
0.1	3.29	No growth.
0.08	2.632	31
0.04	1.316	77
0.01	0.329	100

TABLE IV.

*Relation between Concentration of Methyl Alcohol and Growth of Lupine Roots.*

Concentration of methyl alcohol.		Growth increment, for 24 hrs., expressed as percentage of corresponding increment in control test.
<i>mol per liter</i>	<i>per cent</i>	
1.5	4.8	No growth.
1.0	3.2	27
0.5	1.6	88, 73
0.3	0.96	1.05
0.1	0.32	1.19, 1.16, 1.16
0.05	0.16	1.28
0.01	0.03	76
0.005	0.02	75
0.001	0.003	97

growth at all. It is evident that benzoyl ecgonine, unlike ecgonine itself, is somewhat less toxic than cocaine.

*Effect of Methyl Alcohol.*—The results obtained with methyl alcohol are shown in Table IV. This drug was found to be but little toxic for lupine roots. It required a concentration of 1.5 molar, or 4.8

per cent, to kill the plant, while 1 molar, or 3.2 per cent, gave a growth increment of 27 per cent, or as large as occurred in the controls. Concentrations of 0.1 molar, or 0.32 per cent, gave results suggestive of a stimulation of root elongation.

*Effect of Sodium Benzoate and Methyl Benzoate.*—The results obtained with these compounds were most interesting, as shown in Tables V and VI. It was found that these esters were both very toxic for lupine roots. Solutions of sodium benzoate as dilute as 0.305 molar, or 0.007 per cent, completely inhibit growth, while methyl benzoate produced complete inhibition in concentrations of 0.0001 molar, or 0.014 per cent.

*Effect of Some Mixtures.*—Inasmuch as combinations of drugs in animal experiments often give synergistic or antagonistic results, experiments were made with various mixtures of some of the compounds above considered. Four sets of mixtures were prepared and labeled respectively A, B, C, and D. Mixture A consisted of sodium benzoate, methyl alcohol, and ecgonine hydrochloride, the quantity of each used being one-third of the individual lethal dose. Mixture B consisted of sodium benzoate, methyl alcohol, and ecgonine hydrochloride, the quantity of each used being one-third of the individual lethal dose. Mixture C contained one-half of the lethal doses of ecgonine hydrochloride and methyl benzoate, respectively. Mixture D contained one-half of the lethal doses of benzoyl ecgonine and methyl alcohol, respectively. These mixtures were employed in some experiments in their original concentrations, and in other experiments dilutions of these mixtures were tried. It was found, as might have been expected, that Mixture A produced death of the plants. It was furthermore found that, when Mixture A was diluted to one-half the original concentration, it still produced death; in other words, it gave a synergistic effect. Mixture B was also found to be more toxic than was to be expected from a simple summation of its individual components. On the other hand, Mixtures C and D were found to be slightly less toxic than the arithmetical sum of their component effects; in other words, they suggest a slightly antagonistic action of the components against each other.

TABLE V.

*Relation between Concentration of Sodium Benzoate and Growth of Lupine Roots.*

Concentration of sodium benzoate.		Growth increment, for 24 hrs., expressed as percentage of corresponding increment in control test
<i>mol per liter</i>	<i>per cent</i>	
0.174	2.5	No growth.
0.087	1.25	" "
0.07	1.00	" "
0.04	0.625	" "
0.017	0.25	" "
0.0087	0.125	" "
0.003	0.05	" "
0.0028	0.04	" "
0.002	0.03	" "
0.0015	0.022	" "
0.0014	0.02	" "
0.001	0.0144	" "
0.0005	0.007	" "
0.0003	0.004	11
0.0001	0.001	56.2, 62
0.00005	0.0007	67, 66
0.00001	0.0001	11.1
0.000005	0.00007	0.83
0.000001	0.00001	0.97

TABLE VI.

*Relation between Concentration of Methyl Benzoate and Growth of Lupine Roots.*

Concentration of methyl benzoate.		Growth increment, for 24 hrs., expressed as percentage of corresponding increment in control test.
<i>mol per liter</i>	<i>per cent</i>	
0.001, 0.0001	0.136, 0.014	No growth.
0.00005	0.007	13
0.00001	0.0014	30
0.000005	0.0007	74
0.000001	0.00014	79
0.0000005	0.00007	90, 90
0.0000001	0.000014	75, 93
0.00000005	0.000007	94

## DISCUSSION.

The results obtained with cocaine and its various other compounds on the growth of lupine roots are interesting in themselves, but they become much more so when compared with the effects of the same substances on animal organs and tissues. The results of such a comparison are shown in Table VII. In this table the first column indicates the drugs used, the second column expresses the minimal lethal doses of the same for *Lupinus albus*, while the other columns indicate the corresponding effects on animal tissue. In Column 3 are shown the influence of cocaine and its decomposition products, as well as various other mixtures, on the central nervous system. These results are taken from a study by Macht and Bloom (8) concerning the effects of cocaine on the behavior of white rats in the circular maze. In the fourth column the local anesthetic effects of the various drugs are shown. In Column 5 the effects of cocaine and the other drugs on skeletal muscle are described, the data being taken from Kubota and Macht (9). In Column 6 the toxicity of the various compounds for smooth muscle is indicated. These figures are taken from the as yet unpublished studies of the effects of cocaine on the smooth muscle of the bladder and ureters carried on by Macht and Satani. Finally, the lethal doses of cocaine for cats are given in the last column, the figures being taken from Heffter.<sup>2</sup>

It will be noted that, whereas cocaine is by far the most toxic of the substances studied for animals, it is not nearly so toxic for plants. The lethal dose of cocaine for cats is given as 0.02 to 0.04 gm. per kilo, whereas to produce complete inhibition in the growth of the plant a 2.04 per cent solution is required. Ecgonine hydrochloride is quite toxic for animal tissues and, indeed, in the case of skeletal muscle it paralyzed the contractions of the same in 45 minutes, an effect slightly more toxic than that of cocaine (55 minutes). The lethal dose of ecgonine hydrochloride for lupine roots was comparatively very much smaller than that of cocaine, a concentration of 0.55 per cent being sufficient to kill. Benzoyl ecgonine was much less toxic for lupine roots than ecgonine itself, a result which ran parallel to the compara-

<sup>2</sup>Poulssen, E., in Heffter, A., *Handbuch der experimentellen Pharmakologie*, Berlin, 1920. ii, pt. 1, 145 ff.

TABLE VII.  
*Comparison between Effects of Cocaine and Related Substances on Lupine Roots, Animal Tissues, etc.*

Substance. (1)	Minimal lethal dose for lupine roots. (2)		Effect on central nervous system. (3)	Local anesthetic effect. (4)	Time for paralysis of skeletal muscle. (5)	Effect on smooth muscle. (6)	Lethal dose for cat. (7)
	m	per cent					
Cocaine hydrochloride ..	0.06	2.04	Depression.	Effect was ob- tained	min. 55	1:20,000 1:5,000	gm. per kilo 0.02-0.04
Ecgonine hydrochloride .	0.0025	0.055	None.	None.	45	1:20,000	Over 2.0.
Benzoyl ecgonine.....	0.1	2.5	"	"	Very slight tox- icity.	1:750	1 7
Sodium benzoate .....	0.0005	0.007	"	"	No toxicity.	No effect ob- served.	0
Methyl alcohol.....	1.5	4.8	"	"	"	1:50	4.7
Methyl benzoate.....	0.0001	0.014	"	"	"		
Mixture A.....	(Synergism.)		None.		Toxic.		
Mixture B.....	(Potentiate.)		"				
Mixture C.....	(Antagonism.)		"				
Mixture D.....	( " )				Very slight tox- icity.		

tive toxicity of the two for animals. Methyl alcohol was found to be not very toxic for either animal tissues or lupine roots. The most remarkable results obtained, however, were those with sodium benzoate. This salt is very slightly toxic indeed for animal tissues, so that it is employed as a food preservative, yet it was found to be the most toxic of all the compounds studied for lupine roots. It required a concentration of only 0.007 per cent to kill the plants. Methyl benzoate was also very toxic, being second only to sodium benzoate in that respect.

The reason for the remarkable difference just pointed out, in the toxicity of cocaine for animal and for plant tissues, is as yet unknown. The effects of the drugs studied are certainly not attributable to a simple change in the hydrogen ion concentration of the culture media, for experiments made by the authors showed that the Shive solution may be made more or less acid or alkaline without appreciably affecting the growth of the lupine roots. The value of the pH of the normal Shive solution here used was 6.4. More acid and less acid solutions were prepared by varying the quantity of potassium phosphate used, and it was found that the hydrogen ion concentrations varied from 4.4 to 7.2. Within these limits the growth of lupine roots was very little affected.

#### SUMMARY.

1. The effects of cocaine and its decomposition products were studied on the growth of the young roots of *Lupinus albus*.

2. The results obtained were compared with similar experiments on animal tissues.

3. It was found that, while cocaine is the most toxic of these compounds studied for animal tissues, it was of comparatively low toxicity in respect to its effect on the growth of roots. On the other hand, sodium benzoate, being practically non-toxic for animals, was the most toxic of the compounds studied for the plant roots.

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## STUDIES ON THE REGULATION OF OSMOTIC PRESSURE.

### II. THE EFFECT OF INCREASING CONCENTRATIONS OF ALBUMIN ON THE CONDUCTIVITY OF A SODIUM CHLORIDE SOLUTION.

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(Received for publication, March 23, 1922.)

#### INTRODUCTION.

It has been shown<sup>1</sup> that the addition of gelatin in increasing concentrations to a 0.6 per cent sodium chloride solution affects the conductivity in two ways, depending on the hydrogen ion concentration. At pH 3.3 the conductivity increases with each added increment of gelatin, whereas at pH 5.1 and 7.4 the conductivity decreases as the percentage of gelatin increases. It was suggested that the addition of gelatin to a solution of sodium chloride has two opposite effects: (1) to increase the conductivity by the addition of ionized gelatin, and (2) to decrease the conductivity by the mechanical interference of the undissociated gelatin molecules. The relative value of these two influences seemed to depend on the degree of ionization of the gelatin, which in turn is dependent upon the hydrogen ion concentration. The experimental results indicated that at pH 3.3 the first effect obtained, while at pH 7.4 and 5.1 the second effect was predominant.

This paper presents similar experiments with another protein; *viz.*, egg albumin. A preliminary determination of the conductivities of pure egg albumin solutions varying in concentrations from 0.8 to 8.7 per cent was carried out as in the case of gelatin.

<sup>1</sup> Palmer, W. W., Atchley, D. W., and Loeb, R. F., *J. Gen. Physiol.*, 1920-21, iii, 801.

## EXPERIMENTAL.

The crystalline egg albumin used in these experiments was prepared according to the method described by Hopkins,<sup>2</sup> and recrystallized twice. It was dialyzed until practically free from inorganic salts. The percentage of albumin was determined by drying to constant weight. The hydrogen ion concentration was determined by a gas chain and the conductivity by a Kohlrausch bridge at 25°. Duplicate observations were made in every case. The albumin was brought to the various hydrogen ion concentrations by the addition of NaOH or HCl.

The first experiment (Table I) was performed with pure albumin solutions varying from 1.1 to 8.3 per cent. The conductivity of these

TABLE I.  
*Conductivity of Pure Albumin Solutions.*

Albumin.	Conductivity $\times 10^{-4}$ at pH 3.1.	Albumin.	Conductivity $\times 10^{-4}$ at pH 5.3.	Albumin.	Conductivity $\times 10^{-7}$ at pH 7.3.
<i>per cent</i>		<i>per cent</i>		<i>per cent</i>	
1.6	9.2	1.1	0.9	1.4	2.1
3.2	14.8	2.2	1.5	2.3	2.8
4.8	19.6	3.7	2.3	3.3	4.1
6.4	23.7	4.7	2.8	6.4	7.8
8.0	27.5	7.4	3.8		
		8.3	4.2		

solutions was determined at pH 3.1, 5.3, and 7.3. The results are plotted in Fig. 1, with the concentrations of albumin as abscissæ and the specific conductivities  $\times 10^{-4}$  as ordinates. The curves are reduced to the same scale and plotted at equal intervals above each other. The increase of conductivity with each increment of albumin apparently follows a straight line curve in each instance. The quantitative relations at the various hydrogen ion concentrations are similar to those found in the gelatin solutions.

In a second experiment (Table II) gradually increasing amounts of albumin were added to a 0.6 per cent NaCl solution. Observations

<sup>2</sup> Hopkins, F. G., *J. Physiol.*, 1899-1900, xxv, 306.

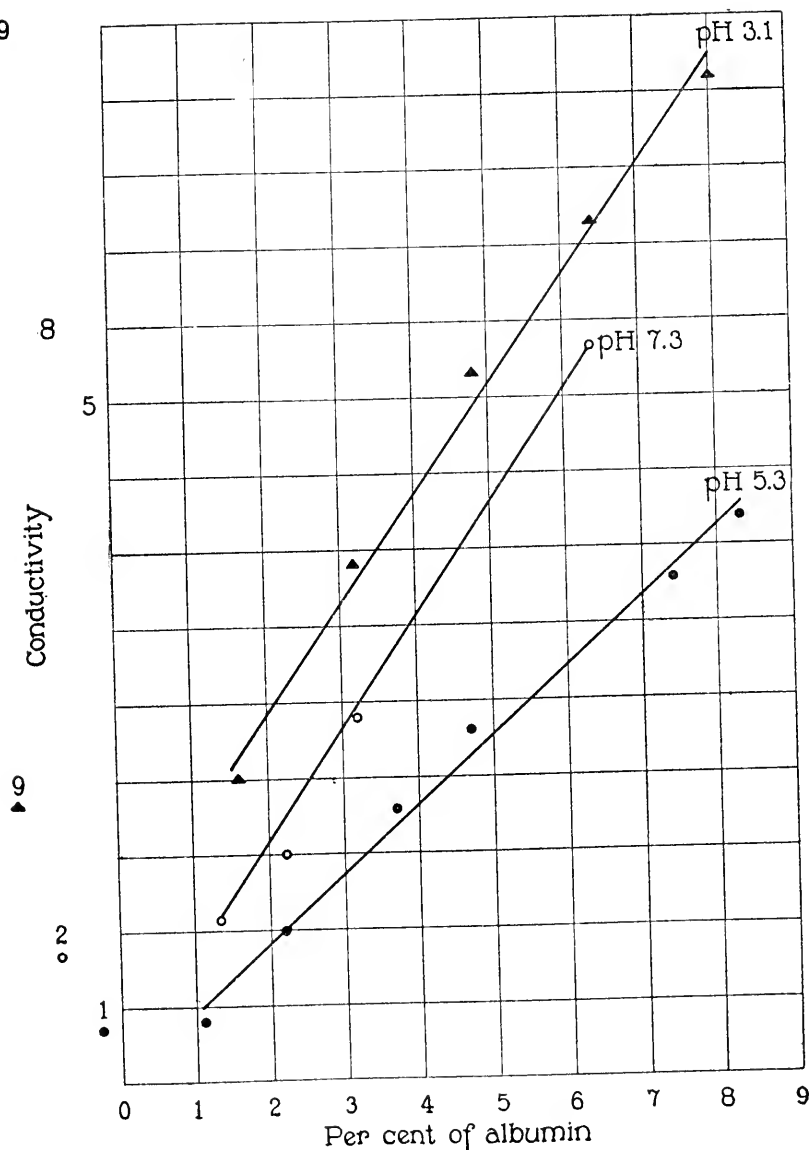


FIG. 1. Conductivities of pure albumin solutions. The abscissæ represent varying concentrations of albumin in per cent, and the ordinates represent specific conductivities  $\times 10^{-4}$ . The increase in conductivity with increasing concentration of albumin is shown; the increase is greatest at pH 3.1, and least near the isoelectric point (5.3). At pH 3.1, the conductivity increases from 9.2 in 1.6 per cent albumin to 27.5 in 8 per cent albumin. At pH 5.3 the conductivity increases from 0.9 to 4.2 with increase in albumin from 1.1 to 8.3 per cent. At pH 7.3 it increases from 2.1 to 7.8 with an increase in albumin from 1.4 to 6.4 per cent.

TABLE II.

*Conductivity of 0.6 Per Cent NaCl Solution with Increasing Albumin.*

Albumin.	Conductivity $\times 10^{-4}$ at pH 3.5.	Albumin.	Conductivity $\times 10^{-4}$ at pH 5.0.	Albumin.	Conductivity $\times 10^{-4}$ at pH 7.3.
<i>per cent</i>		<i>per cent</i>		<i>per cent</i>	
1.1	111.5	0.8	107.9	1.8	110.6
2.9	114.0	1.9	106.4	3.6	108.9
4.8	115.2	3.9	103.8	5.4	107.0
6.6	117.1	5.8	101.2	7.9	105.2
8.3	119.0	8.7	97.0		

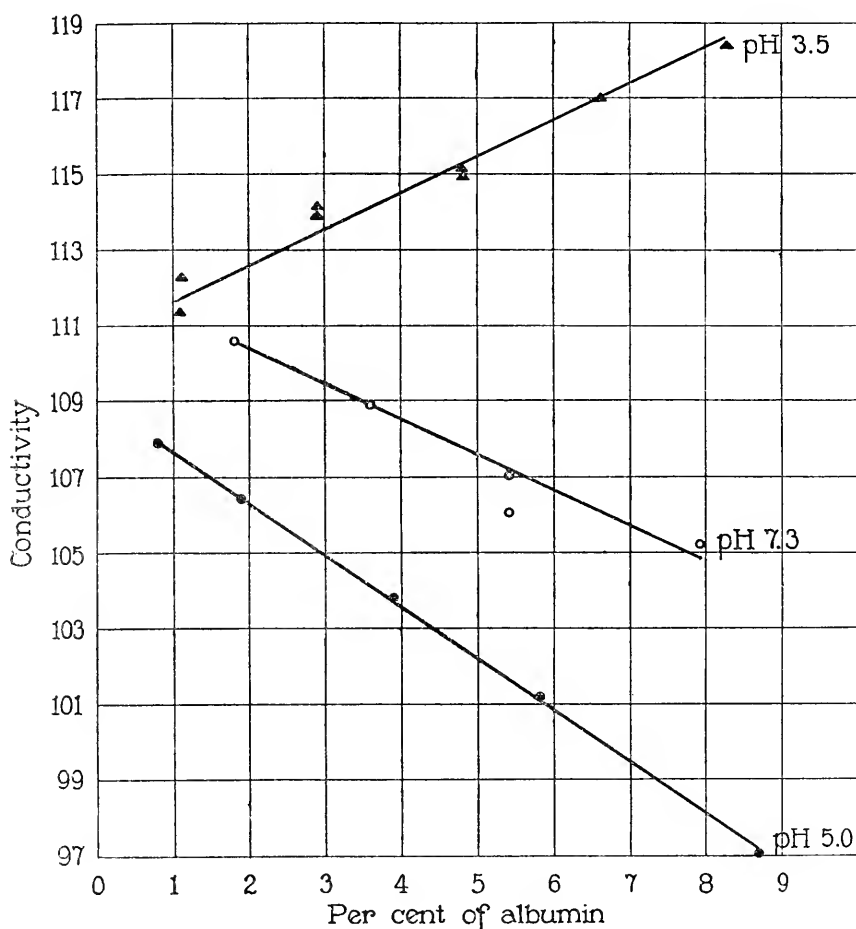


FIG. 2. Changes in specific conductivity ( $\times 10^{-4}$ ) of 0.6 per cent NaCl solutions, with increasing concentration of albumin at various hydrogen ion concentrations. At pH 3.5, conductivity *increases* with increase in albumin per cent. At pH 5.0 and 7.3, conductivity *decreases* with increasing concentration of albumin. This decrease is greater at pH 5.0 than at pH 7.3.

were made at pH 3.5, 5.0, and 7.3. In Fig. 2 the results are plotted on a common scale, conductivities as ordinates and concentrations of albumin as abscissæ. As in the case of pure gelatin solutions, the three curves appear to be straight lines. At pH 3.5 the conductivity increases with the concentration of gelatin, and at pH 5.0 it decreases markedly with increasing amounts of gelatin. At about the reaction of blood, pH 7.3, there is a definite, but less striking, decrease of the conductivity as the percentage of albumin increases.

#### DISCUSSION.

The results charted in Fig. 2 indicate that egg albumin influences the conductivity of a solution of sodium chloride in about the same manner as does gelatin. Considerable support is thereby given to the idea that the factor determining the influence of protein on the conductivity of a NaCl solution is the degree of ionization which is dependent on the pH of the solution. At the reaction of blood, egg albumin is so little ionized that it decreases the conductivity of the salt solution to which it is added.

#### CONCLUSION.

Egg albumin, like gelatin, influences the conductivity of a 0.6 per cent NaCl solution in two ways: (a) At an hydrogen ion concentration of about pH 3.0, increasing concentrations increase the conductivity. (b) Near the isoelectric point of albumin and at the pH of the blood, increasing concentrations of albumin decrease the conductivity of the NaCl solution.



# ON THE EQUILIBRIUM CONDITION BETWEEN BLOOD SERUM AND SEROUS CAVITY FLUIDS.

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## INTRODUCTION.

There has been much speculation concerning the mechanism by which fluid accumulates in the serous cavities of the body. The presence of ascitic fluid in cirrhosis of the liver has been attributed to compression of the portal circulation. In certain types of renal disease, the retention of fluid has been attributed to some disturbance of kidney function. Fluid accumulations in serous cavities, in inflammatory conditions, have been ascribed to an increased permeability of the capillaries. Thus, it appears, that in the minds of investigators, these various types of edema are classified as unrelated phenomena.

We have made a preliminary study of several physicochemical properties of blood sera and edema fluids simultaneously obtained. The cases studied include heart disease and nephritis with ascites, hydrothorax, and subcutaneous edema; cirrhosis of the liver with ascites; and tuberculous pleurisy with effusion. The results seem to indicate that certain constant qualitative relationships exist between blood serum and fluids in the serous cavities (peritoneal and pleural), regardless of the nature of the fluids or the type of disease.

## EXPERIMENTAL.

70 cc. of blood were removed from an arm vein and placed in large centrifuge tubes under oil without exposure to the air. After clotting and centrifugalization, the serum was removed. The patients were tapped immediately after the removal of the blood and the ascitic

TABLE I.

Case.	Diagnosis.	Nature of fluid.	$\Delta$	Specific conductivity $\times 10^{-4}$ .	Molecular concentration of $\text{Cl}^- \times 10^{-3}$ .	Molecular concentration of $\text{HCO}_3^- \times 10^{-3}$ .	Molecular concentration of $\text{Na}^+ \times 10^{-3}$ .	Molecular concentration of $\text{K}^+ \times 10^{-3}$ .	Molecular concentration of $\text{Ca}^{++} \times 10^{-3}$ .	Molecular concentration of glucose $\times 10^{-3}$ .	Molecular concentration of urea $\times 10^{-3}$ .	Non-protein N per 100 cc.	Protein content.
Cl.	Cirrhosis.	Serum.	0.498	113.7	100.0	27.5	124.7	4.2		7.2		mg. 26	6.8
		Ascitic fluid.	0.509	132.5	108.2	26.3	138.2	2.4		7.2		24	0.9
K.	"	Serum.	0.490	117.7	103.8	23.8	133.8	4.7	2.4	8.2	7.8	33	5.2
		Ascitic fluid.	0.488	131.9	109.0	23.8	126.6	2.0	2.3	9.0	8.2	31	0.8
Co.	"	Serum.	0.518	115.9	105.6	26.4	112.2	2.7		7.3		30	7.0
		Ascitic fluid.	0.507	135.2	113.7	25.6	138.6	2.3		9.6		27	0.9
D.	Nephritis.	Serum.	0.535	127.5	110.9	27.0	149.4	3.5		6.3		38	5.0
		Chest fluid.	0.535	144.7	119.7	26.6	147.7	1.7		6.9		34	0.6
P.	Cardiorenal.	Serum.	0.549	125.5	115.3	16.9	139.8	4.7		5.1	19.0	74	6.0
		Ascitic fluid.	0.549	135.8	121.1	16.2	140.8	3.2		5.2	19.0	68	3.3
McF.	Cardiac.	Serum.	0.533	120.6	105.6	28.4	140.0	4.5		5.6		23	7.3
		Ascitic fluid.	0.534	130.0	112.7	28.4	141.3	3.1		6.8		25	4.5
H.	Tuberculous pleurisy.	Serum.	0.515	115.9	101.2	29.9	138.7	4.4		5.5		22	7.1
		Chest fluid.	0.512	121.0	104.4	29.3	142.1	2.7		5.9		21	5.6
Limit of error of analytical methods used.....													
					0.9	0.7	6.1	0.3		0.4	0.3	2.0	



or chest fluid was received under oil, to avoid any marked change in hydrogen ion concentration through loss of  $\text{CO}_2$ . The following physical and chemical determinations were made on the blood sera and the edema fluids; freezing point depression, specific conductivity, Cl,  $\text{HCO}_3$ , Na, K, glucose, non-protein nitrogen, protein per cent (by Kjeldahl and refractivity), and, in certain cases, urea and Ca. A description of the methods employed, and other details, will be published later. The results of these observations are collected in Table I.

In four cases, serum was placed in a thin collodion sac with a capacity of about 6 cc. and immersed in a bottle containing 250 cc. of edema fluid. This bottle was kept at  $25^\circ\text{C}$ . A manometer was placed in

TABLE II.

Case.	Diagnosis.	Molecular concentration of K $\times 10^{-3}$ in fluid.	Molecular concentration of K $\times 10^{-3}$ in serum.		Molecular concentration of Cl $\times 10^{-3}$ in fluid.	Molecular concentration of Cl $\times 10^{-3}$ in serum.		Protein in serum.	
			Before dialysis.	After dialysis.		Before dialysis.	After dialysis.	Before dialysis.	After dialysis.
								<i>per cent</i>	<i>per cent</i>
P.	Cardiorenal.	3.2	4.7	5.2				6.0	5.9
Co.	Cirrhosis.	2.3	2.7	2.6	113.7	105.6	107.2	7.0	6.7
H.	Tuberculous pleurisy.	2.7	4.4	3.9					
McF.	Cardiac.	3.1	4.5	4.6	112.7	105.6	109.8		

the collodion sac, and the serum level was so adjusted that there would be little or no change in level with the establishment of equilibrium. After 18 hours, the contents of the sac were analyzed for protein per cent, K, and Cl. The results of these analyses were compared with the original concentrations and with those of the edema fluid as shown in Table II. The bottle containing the edema fluid and collodion sac was kept closed with a rubber stopper and a soda-lime tube to prevent change in hydrogen ion concentration.

## DISCUSSION.

From the data in Table I, the following relationships between blood serum and edema fluids are apparently constant. (a) The freezing

point depression of serum and of edema fluid is the same—within the limit of error of the method when applied to physiological solutions. (b) The conductivity of the edema fluid is always higher than that of the blood, but the greater the protein content of the edema fluid, the closer the conductivity approaches that of the serum. This is in accord with experiments on pure protein solutions, in which it was found that, at the pH of the body fluids, the higher the protein content, the lower the conductivity.<sup>1</sup> (c) The chloride content of the edema fluid is always higher than that of the serum. (This is at variance with findings of Epstein.<sup>2</sup>) This difference of Cl concentration in blood and edema fluids diminishes, in general, as the protein content of the edema fluid increases. (d) The concentration of potassium is greater in the serum than in the edema fluid. (e) The concentrations of  $\text{HCO}_3$ , Na, sugar, non-protein nitrogen, and Ca and urea, where these were determined, are approximately the same, in the determinations made thus far.

The experiments on the dialysis of serum against edema fluid reported in Table II, demonstrate that no new equilibrium is established when the two fluids are separated by a simple collodion membrane. The relatively high concentration of potassium inside and the relatively high concentration of chlorine outside the membrane are but slightly changed. The results suggest that these interesting relationships depend on a simple membrane equilibrium and are not entirely due to properties peculiar to living protoplasm.

#### CONCLUSIONS.

1. Comparative studies of blood serum and edema fluid from the same individual indicate that, regardless of the pathological condition present, whether the fluid be "transudate" or "exudate," certain definite qualitative chemical relations obtain.

2. The chief feature of these relations is that the edema fluid contains more Cl and less K than the blood serum; while the Na,  $\text{HCO}_3$ , Ca, urea, glucose, and non-protein nitrogen exist in approximately

<sup>1</sup> Palmer, W. W., Atchley, D. W., and Loeb, R. F., *J. Gen. Physiol.*, 1920-21, iii, 801; 1921-22, iv, 585.

<sup>2</sup> Epstein, A. A., *J. Exp. Med.*, 1914, xx, 334.

the same concentrations in the serum and in the edema fluid. The freezing point is also the same in both fluids, while the specific conductivity is constantly higher in the edema fluid.

3. The above mentioned variations between the edema fluid and the serum appear to be related to the difference in the concentration of protein in the two solutions.

4. The relationships between blood serum and edema fluid seem to result from a simple membrane equilibrium, influenced in part by the proteins present.



# THE COLLOIDAL BEHAVIOR OF EDESTIN.

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## I.

### INTRODUCTION.

It has been shown in a series of papers by Loeb<sup>1</sup> that the physical, chemical, and so-called colloidal properties of solutions of the proteins, gelatin, egg albumin, and casein, can be simply explained by two general principles. The first of these is that proteins are amphoteric electrolytes, reacting stoichiometrically with acids and bases to form salts capable of electrolytic dissociation; the second is the principle of Donnan's membrane equilibrium,<sup>2</sup> which is set up when two solutions are separated by a membrane impermeable to one ion of one of the solutions.

The present investigation was undertaken with the object of finding out whether these laws would explain the behavior of solutions of a protein of a different class; namely, a globulin. The globulin selected for the purpose was edestin; and it was found that its solutions obeyed the same laws which had been shown to apply in the case of the other proteins.

The edestin used in these experiments was prepared from ground hemp-seed by the method of Osborne,<sup>3,4</sup> with slight modifications. The hemp-seed meal was extracted three times with 10 per cent sodium chloride solution at 60°C., without previously extracting the oil. The edestin was precipitated by dilution and recrystallized once from sodium chloride solution according to Osborne. The substance was

<sup>1</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922; *J. Gen. Physiol.*, 1918-22, i-iv.

<sup>2</sup> Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572.

<sup>3</sup> Osborne, T. B., *J. Am. Chem. Soc.*, 1902, xxiv, 28, 39.

<sup>4</sup> Osborne, T. B., *Abderhalden's Handb. d. biochem. Arbeitsmethoden*, 1910, ii, 289.

freed from sodium chloride by washing repeatedly with 50 per cent alcohol, and was partly dried by washing with absolute alcohol and then with ether, which left it with a moisture content of 10.1 per cent as determined by heating to constant weight at 110°C. The dry powder was found to contain 18.4 per cent of nitrogen, as determined by a micro-Kjeldahl method accurate to about 1 per cent, whereas Osborne<sup>3</sup> reported 18.69 per cent of nitrogen. Under the microscope the preparation appeared to have the form of fragments of crystals.

The isoelectric point of edestin was given by Rona and Michaelis<sup>5</sup> as at a hydrogen ion concentration of  $1.3 \times 10^{-7}$  (pH 6.89). This was the point of maximum precipitation obtained by using a series of phosphate buffers.<sup>6</sup> Attempts were made to determine the isoelectric point of the present preparation by similar methods, but the point of maximum precipitation seemed to vary with the buffer used. Most of the values obtained, however, lay between pH 5 and 6. Measurements of the osmotic pressure developed in collodion bags by suspensions of edestin in various concentrations of very dilute sodium hydroxide indicated a minimum between pH 5 and 6. Electrophoresis experiments with suspensions of this edestin were made by Dr. John H. Northrop of this laboratory, who also obtained different results with different buffers, which indicated, however, that the isoelectric point appeared to lie between pH. 5.5 and 6.0. The pH of suspensions of this edestin in distilled water was found to be 5.0.

## II.

### *Titration of Edestin with Acids and Bases.*

Titration curves of solutions of edestin in acids and bases were obtained in the region where 0.45 gm. could be almost completely dissolved in 100 cc.; *i.e.*, below pH 5.0 and above pH 9.0. Solutions were prepared containing different quantities of acid or alkali but of the same concentration with respect to edestin, and the pH values were

<sup>5</sup>Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193. The edestin used by Rona and Michaelis was apparently a different substance from the present preparation, since they stated that only a small fraction of 1 per cent was soluble in 0.1 N  $\text{H}_3\text{PO}_4$ , while the edestin used in this investigation was completely soluble to the extent of 1 per cent or more in 0.1 N  $\text{H}_3\text{PO}_4$ .

<sup>6</sup>See also Michaelis, L., and Mendelssohn, A., *Biochem. Z.*, 1914, lxxv, 1. Using acetate buffers they obtained a value of  $2.5 \times 10^{-6}$  (pH 5.60) for the isoelectric point of a different edestin preparation.

ascertained by means of the hydrogen electrode, using a salt bridge of saturated potassium chloride and a saturated potassium chloride calomel cell. The measurements were made at  $33^\circ \pm 0.5^\circ$ , and were referred to 0.1 M HCl as a standard, its pH being taken as 1.036.

The titration curve obtained with 0.45 per cent solutions of edestin in phosphoric acid is given in Fig. 1, Curve I. As a means of determining how much of the acid was combined with the edestin, Curve II was obtained by measuring the pH of mixtures of phosphoric acid and water alone. Curve III, which gives the amount of phosphoric acid apparently combined with the edestin, was obtained by subtracting the ordinates of Curve II from those of Curve I at corresponding pH values. Since phosphoric acid was known to be a weak acid, it was suspected that some of the acid indicated by Curve III to be combined with the edestin might really be present as un-ionized molecules, due to the common ion effect of the ionized edestin phosphate. Accordingly a calculation was made of the first ionization constant of phosphoric acid at  $33^\circ$ , from the data used in plotting Curve II. The results are as follows:

pH	$k = \frac{[H^+][H_2PO_4^-]}{[H_3PO_4]}$
2.72	0.0101
2.47	0.0100
2.29	0.0094
2.18	0.0092
1.93	0.0096

These values may be compared with that found by Abbott and Bray at  $18^\circ$ , 0.011.<sup>7</sup> Their constancy indicates that over this range of pH the acid is really monobasic. Accordingly this ionization constant, roughly 0.01, was used to calculate the amount of edestin phosphate actually present, assuming complete ionization of this salt. This was done as follows:

Let  $c$  = total concentration of  $H_3PO_4$  (ionized, un-ionized, and present as edestin phosphate),

$x$  = concentration of  $H_2PO_4^-$  from edestin phosphate (assumed to be completely ionized),

$h$  = concentration of  $H^+$  = concentration of  $H_2PO_4^-$  from  $H_3PO_4$ ,

$k$  = primary ionization constant of  $H_3PO_4$ ,

<sup>7</sup> Abbott, G. A., and Bray, W. C. *J. Am. Chem. Soc.*, 1909, **xxxi**, 760.

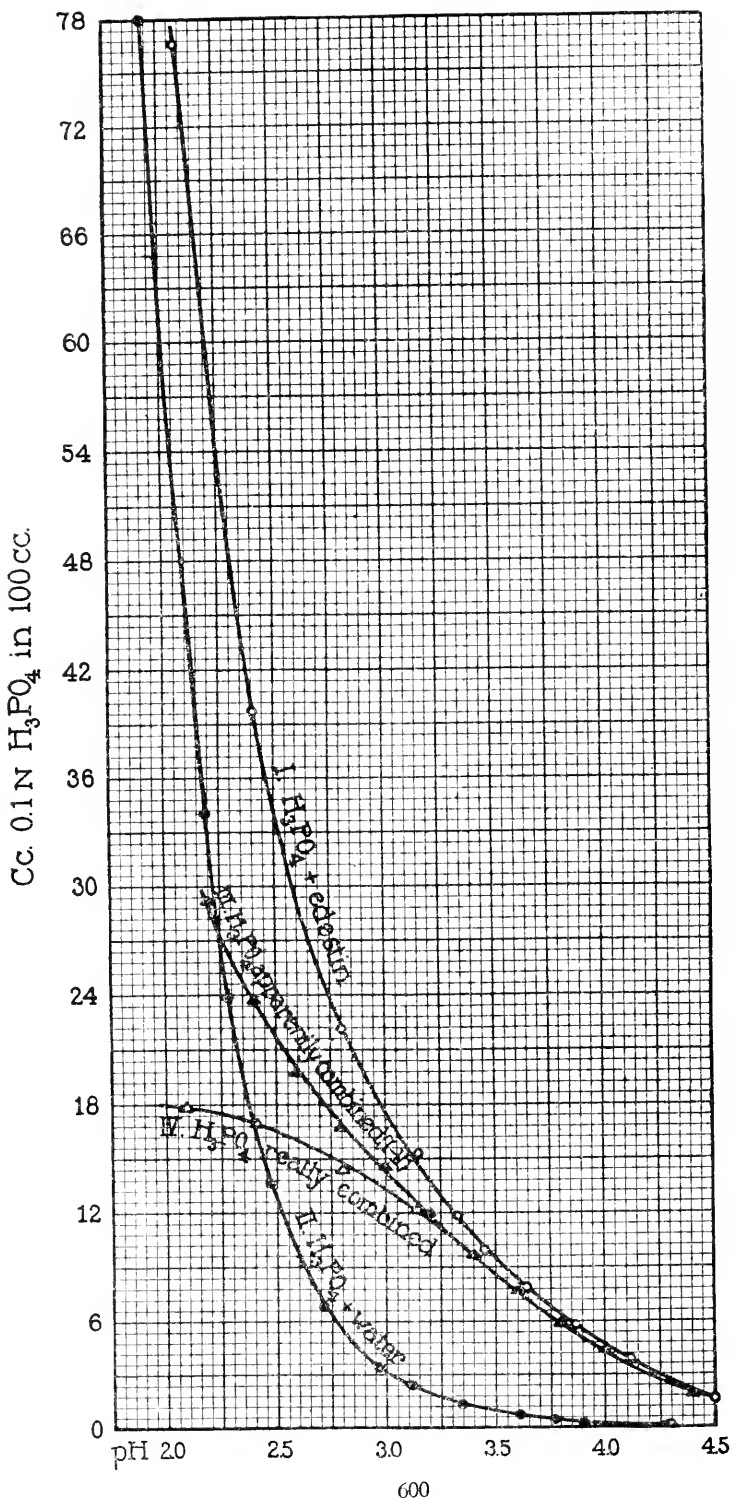


FIG. 1. I. Titration curve of 0.45 per cent edestin with 0.1 N  $\text{H}_3\text{PO}_4$ . II. Titration curve of water with 0.1 N  $\text{H}_3\text{PO}_4$ . III. Difference between I and II. IV. Amounts of  $\text{H}_3\text{PO}_4$  combined with edestin, calculated from I and ionization constant of  $\text{H}_3\text{PO}_4$ .



Then

$$k = \frac{[\text{H}^+][\text{H}_2\text{PO}_4^-]}{[\text{H}_3\text{PO}_4]} = \frac{h(h+x)}{c-h-x}$$

$$x = \frac{kc}{h+k} - h$$

The values of  $x$  obtained in this way, expressed in cc. of 0.1 N  $\text{H}_3\text{PO}_4$  per 100 cc., are plotted in Curve IV, which indicates the amount of  $\text{H}_3\text{PO}_4$  really combined with the edestin. It was found that above pH 3.4 the values so calculated did not differ much from those plotted in Curve III, although it is not strictly justifiable to consider  $\text{H}_3\text{PO}_4$  as a monobasic acid in this range of pH, since the values obtained for  $k$  were not constant above pH 3.

Curves representing the amounts of hydrochloric, sulfuric, and oxalic acids combined with 0.45 gm. of edestin in 100 cc. are given in Fig. 2, along with the curve for  $\text{H}_3\text{PO}_4$  as given in Fig. 1, Curve IV. In the case of these stronger acids the amount combined was obtained in the same way as Curve III, Fig. 1, by subtracting the ordinates of the acid-water curve from those of the acid-destin curve. The curves for HCl and  $\text{H}_2\text{SO}_4$  are nearly identical, indicating that each combines in equivalent proportions with edestin; *i.e.*,  $\text{H}_2\text{SO}_4$  acts as a dibasic acid. The curve for  $\text{H}_2\text{C}_2\text{O}_4$  above pH 4 is identical with that for  $\text{H}_2\text{SO}_4$ , indicating that here oxalic acid is also dibasic. The curves soon diverge, however, and at the maximum that for oxalic acid is almost exactly twice as high as that for hydrochloric, indicating that here oxalic acid combines not in equivalent but in molecular proportions; *i.e.*, it is here a monobasic acid. The curve for  $\text{H}_3\text{PO}_4$  seems to reach a maximum at a height a little over three times that of the curve for HCl and  $\text{H}_2\text{SO}_4$ , indicating that  $\text{H}_3\text{PO}_4$  is combined with the edestin in molecular, not in equivalent proportions, or that  $\text{H}_3\text{PO}_4$  combines with edestin as a monobasic acid.

In order to show how closely these proportions hold, and in what range of pH, the curves of Fig. 2 were plotted on a large scale and the values given in Table I were read off.

The values in Table I show that the ratios are most nearly as 1:2:3 in the region where the curves become horizontal, indicating that the edestin is all combined.

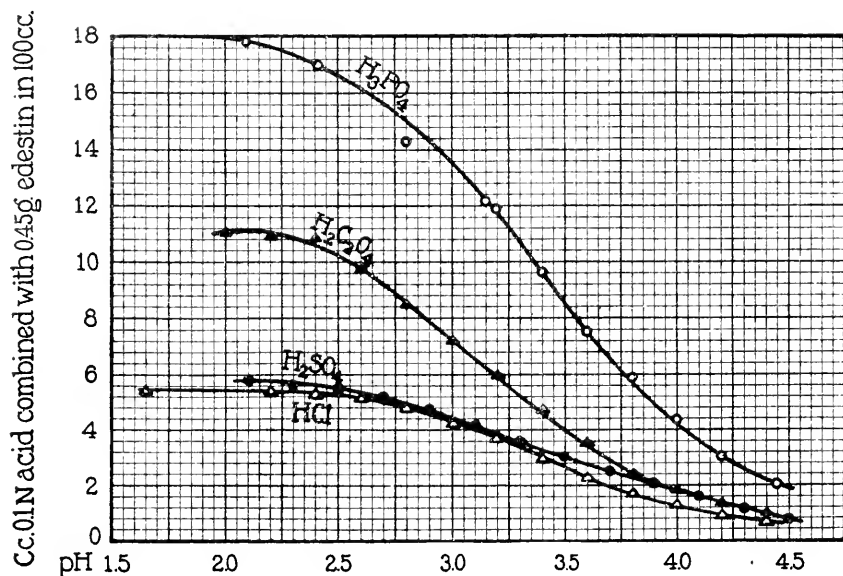


FIG. 2. Amounts of 0.1 N acid combined with 0.45 gm. edestin in 100 cc. Values for HCl,  $\text{H}_2\text{SO}_4$ , and  $\text{H}_2\text{C}_2\text{O}_4$  obtained by difference between titration curves with and without protein. Values for  $\text{H}_3\text{PO}_4$  obtained by calculation, as in Fig. 1, IV.

TABLE I.

*Ratios of Amounts of Different Acids Combined with Edestin at Corresponding pH Values.*

pH	HCl	$\text{H}_2\text{SO}_4$	$\text{H}_2\text{C}_2\text{O}_4$	$\text{H}_3\text{PO}_4$	Ratio $\frac{\text{H}_2\text{SO}_4}{\text{HCl}}$	Ratio $\frac{\text{H}_2\text{C}_2\text{O}_4}{\text{HCl}}$	Ratio $\frac{\text{H}_3\text{PO}_4}{\text{HCl}}$
	cc.	cc.	cc.	cc.			
2.1	5.6	5.8	10.8	17.8	1.0	1.9	3.2
2.4	5.3	5.6	10.8	17.0	1.1	2.0	3.2
2.7	5.05	5.2	9.15	15.4	1.0	1.8	3.0
3.0	4.3	4.4	7.2	13.0	1.0	1.7	3.0
3.3	3.35	3.6	5.35	10.1	1.1	1.6	3.0
3.6	2.3	2.8	3.5	6.95	1.2	1.5	3.0
3.9	1.5	2.05	2.1	4.2	1.3	1.4	2.8
4.2	0.95	1.35	1.3	2.1	1.4	1.4	2.2
4.5	0.6	0.75	0.85	0.8	1.3	1.4	1.3

Attempts were made to carry these curves farther into the region of lower pH, but since they are obtained from differences between the ordinates of steep curves like I and II in Fig. 1, the errors become too

great, as the experimental error in the pH determination is about  $\pm 0.02$  pH.

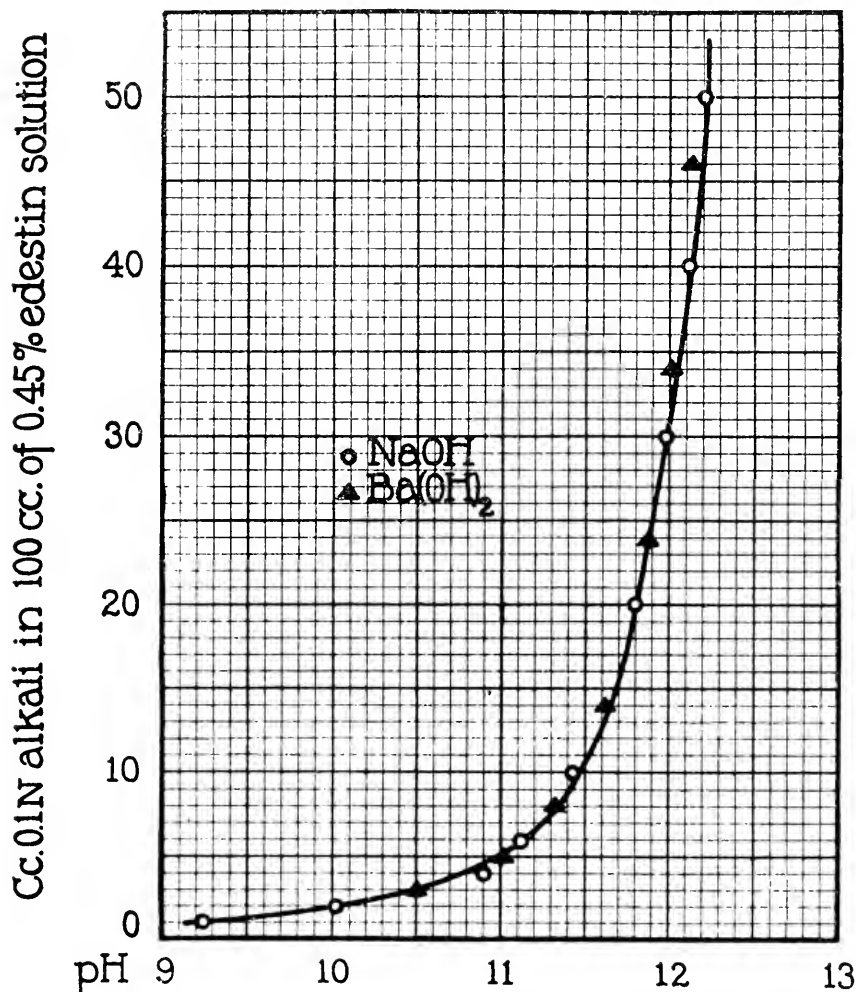


FIG. 3. Titration curve of 0.45 per cent edestin with 0.1 N NaOH and Ba(OH)<sub>2</sub>.

Fig. 3 represents the titration of 0.45 per cent edestin solutions with 0.1 N NaOH and Ba(OH)<sub>2</sub>. The curve is the original titration curve, and does not represent the amount of alkali combined with the edestin. However, since the points fall on one curve, it seems fairly

evident that the edestin must be combined with these strong alkalies in chemically equivalent proportions.

It should be pointed out that these titration experiments with edestin and acids or bases are completely analogous to those previously obtained by Loeb<sup>1</sup> with gelatin, casein, and egg albumin, and that they are in complete agreement with the idea that proteins are amphoteric electrolytes, reacting chemically and stoichiometrically with acids and bases.

### III.

#### *Membrane Potentials.*

Loeb<sup>8,9,10</sup> showed that when a solution of gelatin or egg albumin in dilute acid was separated by a collodion membrane from an aqueous solution of the same acid, containing no protein, a difference in electrical potential existed between the two solutions. Moreover, he found it possible to calculate the magnitude of the potential difference with considerable accuracy from the hydrogen ion concentrations of the two solutions, on the basis of Donnan's theory,<sup>2</sup> according to which, for 25°C.,

$$\text{P.D.} = 59 \log \frac{x}{y} \text{ millivolts} \quad (1)$$

where  $x$  represents the hydrogen concentration of the outside solution and  $y$  that of the inside solution. He showed also that the presence of increasing concentrations of a neutral salt decreased the P.D., the decrease being dependent on the concentration and valence of the anion.

Experiments were carried out to determine whether similar results could be obtained with edestin. Solutions were prepared containing 0.45 gm. of edestin in 100 cc. of HCl of concentration sufficient to give a pH of 3, and containing varying concentrations of Na<sub>2</sub>SO<sub>4</sub>, NaCl, CaCl<sub>2</sub>, or LaCl<sub>3</sub>. These were placed in 50 cc. collodion bags fitted with rubber stoppers and manometer tubes, and the bags were suspended in beakers of HCl of pH 3, which had been made up to

<sup>8</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 557.

<sup>9</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 667.

<sup>10</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 351.

have the same salt concentration as the protein solutions. The beakers were set in a water thermostat at  $25^{\circ} \pm 1^{\circ}\text{C}$ . After equilibrium had been established (12 to 48 hours), the osmotic pressure was measured in terms of millimeters of the solution in the manometer tubes. The P.D. between the inside and outside solutions was determined in each case with the aid of saturated KCl calomel electrodes and a Compton electrometer. The P.D. measurements were made in a room at about  $20^{\circ}\text{C}$ ., but the solutions were very nearly at  $25^{\circ}$ , since the P. D. was determined for each solution within 2 or 3 minutes after removing it from the thermostat at  $25^{\circ}$ . Finally the pH of the inside and outside solutions, at  $33^{\circ}$ , was determined with the hydrogen electrode and potentiometer. The calculated P. D. values were reduced to  $25^{\circ}$  by multiplying by  $\frac{330}{300}$  the differences between the E.M.F. readings obtained for the two solutions with the hydrogen electrode. (This amounts to the same thing as using equation (1), but avoids slight arithmetical errors due to rounding off the values for pH.) The P.D. values were read to 0.5 millivolts, but the reproducibility was of the order of 1 millivolt.

The results of experiments with 0.45 per cent edestin chloride, at pH 3, and the four salts mentioned, are given in Tables II to V.

The excellent agreement of the observed and calculated values for the P.D. in these salt experiments proves that the Donnan equilibrium governs the effect of salt on the P.D. of edestin chloride solutions fully as well as in the case of gelatin or albumin chloride. Tables II and III show that here too it is the anion of the salt which has the depressing effect on the P.D., the sulfate ion being more effective than the chloride ion. An increase in the concentration of an ion of opposite charge to that of the protein ion tends to prevent the forcing of acid from the inside to the outside solution, and hence decreases the difference in pH and the resulting P.D. This may also be shown clearly from the results with the different chlorides by plotting the P.D. against the equivalent concentration of chloride ion furnished by the salt, assuming complete or equal ionization of the inorganic chlorides. The results in Tables III, IV, and V are so plotted in Fig. 4. It is evident that the effects of NaCl,  $\text{CaCl}_2$ , and  $\text{LaCl}_3$  are identical, if compared at the same concentration of chloride ion. This proves beyond a doubt that the valence or nature of the cation is of no influence on the P.D. of edestin chloride.

TABLE II.

*Effect of Sodium Sulfate on the P.H. and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of Na <sub>2</sub> SO <sub>4</sub> .	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	M/8,192	M/16,384	0
pH inside.....	Edestin precipitated.	2.93	2.86	2.83	2.88	2.90	2.93	2.97	2.99	3.00
pH outside.....		2.92	2.83	2.78	2.77	2.74	2.72	2.70	2.69	2.69
Observed P.D., millivolts ..		0.5	2.0	4.0	4.5	9.5	13.5	16.0	17.0	19.0
Calculated P.D., millivolts ..		0	1.5	3.5	7.0	9.5	12.5	15.5	17.0	18.0
Observed osmotic pressure, mm.....		6	10	24	48	71	106	144	172	204

TABLE III.

*Effect of Sodium Chloride on the P.H. and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of NaCl.	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	0
pH inside.....	2.79	2.78	2.79	2.77	2.84	2.84	2.89	2.89	2.95	3.00
pH outside.....	2.79	2.77	2.77	2.74	2.77	2.74	2.75	2.70	2.72	2.70
Observed P.D., millivolts ..	0	0.5	1.0	2.0	3.0	6.5	8.0	10.5	14.0	17.5
Calculated P.D., millivolts ..	0	1.0	1.0	2.0	4.0	6.0	8.0	10.5	13.5	17.5
Observed osmotic pressure, mm.....	8	17	27	34	41	56	88	124	152	201

TABLE IV.

*Effect of Calcium Chloride on the P.H. and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of CaCl <sub>2</sub> .	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	0
pH inside.....	2.82	2.80	2.80	2.88	2.82	2.87	2.92	2.95	2.98	3.02
pH outside.....	2.82	2.78	2.78	2.78	2.75	2.75	2.72	2.70	2.72	2.70
Observed P.D., millivolts ..	0	0.5	1.5	3.0	5.0	8.0	11.0	14.0	15.5	15.5
Calculated P.D., millivolts ..	0	1.0	1.0	6.0	4.0	7.0	11.5	14.5	15.5	18.5
Observed osmotic pressure, mm.....	19	26	34	42	55	83	121	151	177	205

TABLE V.

*Effect of Lanthanum Chloride on the p.D. and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of LaCl <sub>3</sub> .	M/24	M/48	M/96	M/192	M/384	M/768	M/1,536	M/3,072	M/6,144	0
pH inside.....	2.79	2.79	2.80	2.79	2.80	2.85	2.87	2.95	2.98	3.00
pH outside.....	2.78	2.78	2.77	2.75	2.74	2.74	2.70	2.70	2.70	2.69
Observed p.D., millivolts ..	0	0.5	1.5	3.5	4.5	6.5	10.5	13.5	15.5	18.5
Calculated p.D., millivolts ..	0.5	0.5	2.0	2.5	4.0	7.0	10.0	14.5	16.5	18.5
Observed osmotic pressure, mm.....	20	26	33	41	54	82	117	154	180	199

TABLE VI

## CORRECTION.

On page 606, Vol. iv, No. 5, in the italic headings for Tables II, III, and IV for P. H. read P. D.

pH inside.....	1.47	2.02	2.51	2.92	3.30	3.69	4.20	4.73	5.09
pH outside.....	1.43	1.90	2.29	2.62	2.98	3.38	3.94	4.48	4.81
Observed P. D., millivolts.....	2.0	7.5	12.5	17.0	18.0	16.5	12.5	8.0	4.5
Calculated P. D., millivolts.....	2.0	7.5	12.5	17.5	18.5	18.5	15.5	14.5	16.5
Observed osmotic pressure, mm..	82	191	328	383	331	246	134	65	28

TABLE VIII.

*Effect of pH on p.D. and Osmotic Pressure of 1 Per Cent Edestin Acetate in M/100 Sodium Acetate.*

pH inside.....	3.25	3.45	3.57	3.74	3.94	4.10	4.21	4.39	4.51	4.67
pH outside.....	3.12	3.35	3.48	3.64	3.86	4.04	4.15	4.34	4.46	4.62
Observed p.D., millivolts.....	6.5	6.0	5.5	5.0	4.5	4.5	3.5	3.0	2.5	2.5
Calculated p.D., millivolts.....	7.5	6.0	5.5	6.5	4.5	3.5	3.5	3.0	3.0	2.5
Observed osmotic pressure, mm..	139	114	102	88	71	62	56	49	44	38

TABLE II.

*Effect of Sodium Sulfate on the P.H. and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of Na <sub>2</sub> SO <sub>4</sub> .	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	M/8,192	M/16,384	0
pH inside.....	Edestin precipitated.	2.93	2.86	2.83	2.88	2.90	2.93	2.97	2.99	3.00
pH outside.....		2.92	2.83	2.78	2.77	2.74	2.72	2.70	2.69	2.69
Observed P.D., millivolts ..		0.5	2.0	4.0	4.5	9.5	13.5	16.0	17.0	19.0
Calculated P.D., millivolts ..		0	1.5	3.5	7.0	9.5	12.5	15.5	17.0	18.0
Observed osmotic pressure, mm.....		6	10	24	48	71	106	144	172	204

sure, mm.....

TABLE IV.

*Effect of Calcium Chloride on the P.H. and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of CaCl <sub>2</sub> .	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	0
pH inside.....	2.82	2.80	2.80	2.88	2.82	2.87	2.92	2.95	2.98	3.02
pH outside.....	2.82	2.78	2.78	2.78	2.75	2.75	2.72	2.70	2.72	2.70
Observed P.D., millivolts ..	0	0.5	1.5	3.0	5.0	8.0	11.0	14.0	15.5	15.5
Calculated P.D., millivolts ..	0	1.0	1.0	6.0	4.0	7.0	11.5	14.5	15.5	18.5
Observed osmotic pressure, mm.....	19	26	34	42	55	83	121	151	177	205



TABLE V.

*Effect of Lanthanum Chloride on the P.D. and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of LaCl <sub>3</sub> .	M/24	M/48	M/96	M/192	M/384	M/768	M/1,536	M/3,072	M/6,144	0
pH inside.....	2.79	2.79	2.80	2.79	2.80	2.85	2.87	2.95	2.98	3.00
pH outside.....	2.78	2.78	2.77	2.75	2.74	2.74	2.70	2.70	2.70	2.69
Observed P.D., millivolts ..	0	0.5	1.5	3.5	4.5	6.5	10.5	13.5	15.5	18.5
Calculated P.D., millivolts ..	0.5	0.5	2.0	2.5	4.0	7.0	10.0	14.5	16.5	18.5
Observed osmotic pressure, mm.....	20	26	33	41	54	82	117	154	180	199

TABLE VI.

*Effect of pH on P.D. and Osmotic Pressure of 1 Per Cent Edestin Chloride.*

pH inside.....	1.47	2.04	2.59	3.06	3.44	3.91	4.38	4.78	5.09
pH outside.....	1.43	1.91	2.32	2.61	2.90	3.23	3.61	3.91	4.15
Observed P.D., millivolts.....	5.0	7.5	14.0	23.0	29.0	39.0	42.5	37.0	32.5
Calculated P.D., millivolts.....	2.0	8.0	16.0	26.0	32.0	40.0	46.0	52.0	56.0
Observed osmotic pressure, mm..	94	239	415	535	526	501	390	270	200

TABLE VII.

*Effect of pH on P.D. and Osmotic Pressure of 1 Per Cent Edestin Chloride in M/512 Sodium Chloride.*

pH inside.....	1.47	2.02	2.51	2.92	3.30	3.69	4.20	4.73	5.09
pH outside.....	1.43	1.90	2.29	2.62	2.98	3.38	3.94	4.48	4.81
Observed P. D., millivolts.....	2.0	7.5	12.5	17.0	18.0	16.5	12.5	8.0	4.5
Calculated P. D., millivolts.....	2.0	7.5	12.5	17.5	18.5	18.5	15.5	14.5	16.5
Observed osmotic pressure, mm..	82	191	328	383	331	246	134	65	28

TABLE VIII.

*Effect of pH on P.D. and Osmotic Pressure of 1 Per Cent Edestin Acetate in M/100 Sodium Acetate.*

pH inside.....	3.25	3.45	3.57	3.74	3.94	4.10	4.21	4.39	4.51	4.67
pH outside.....	3.12	3.35	3.48	3.64	3.86	4.04	4.15	4.34	4.46	4.62
Observed P.D., millivolts.....	6.5	6.0	5.5	5.0	4.5	4.5	3.5	3.0	2.5	2.5
Calculated P.D., millivolts.....	7.5	6.0	5.5	6.5	4.5	3.5	3.5	3.0	3.0	2.5
Observed osmotic pressure, mm..	139	114	102	88	71	62	56	49	44	38

The effect of the hydrogen ion concentration on the P.D. was determined by using 1 per cent solutions of edestin in varying concentrations of HCl without the addition of salt. The results of such an experiment are given in Table VI and Fig. 5.

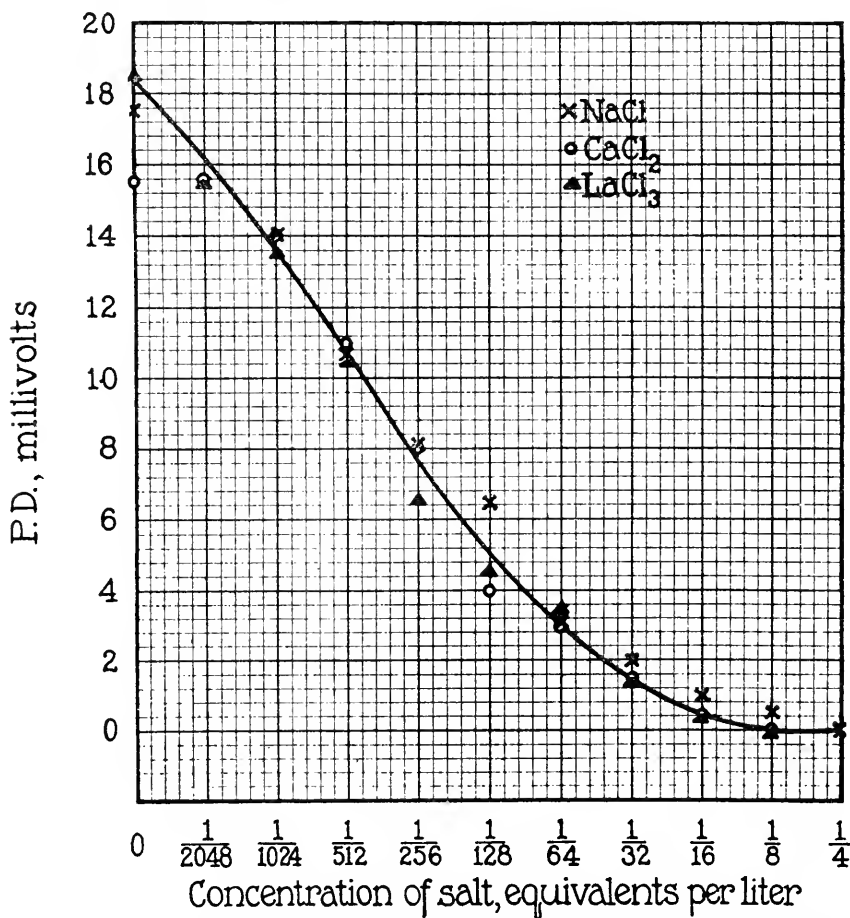


FIG. 4. Effect of different chlorides on P.D. observed with 0.45 per cent edestin chloride at pH 3.

It will be noticed that the observed and calculated values for P.D. agree fairly well up to pH 4.0. The results therefore show that Donnan's theory does apply quantitatively to edestin chloride solutions between pH 1.5 and 4.0.

Since it had been noticed that the agreement between observed and calculated P.D. was better when the solution had a higher concentration of electrolyte, the experiment of Table VI was repeated with all solutions  $M/512$  with respect to NaCl. The results are given in Table VII.

The agreement with the theory is excellent up to pH 3, but again becomes poor above pH 4. In comparing Tables VI and VII it may be observed that the presence of  $M/512$  NaCl had more effect in decreasing the P.D. of those solutions which contained less HCl. This

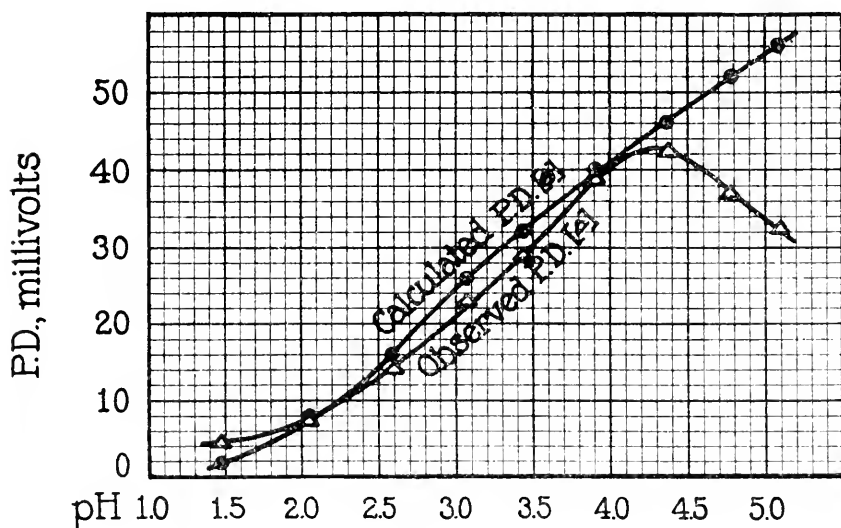


FIG. 5. Effect of pH on P.D. observed and calculated for 1 per cent edestin chloride.

is in line with the experiments already described on the effect of salt concentration on the P.D., indicating that the depression of the P.D. is due to the anion.

In order to obviate any error in the calculated P.D. which might be due to difficulty in determining accurately the pH of the very dilute acid in some of the outside solutions, an experiment was run in which both inside and outside solutions were buffered by  $M/100$  sodium acetate and different concentrations of acetic acid. The results are given in Table VIII and Fig. 6.

It will be observed that the agreement between the observed and calculated values for the P.D. is within the experimental error, and that the experiment includes that range of pH where the values did not agree well in the case of the solutions without buffer. The curve in Fig. 6 is of a different shape from that in Fig. 5 because the depressing action of the anion of the salt has more effect in the less acid solutions, since the concentration of the anion furnished by the acid is originally less in such solutions. The existence of a difference in pH in such buffered solutions and the agreement between the observed

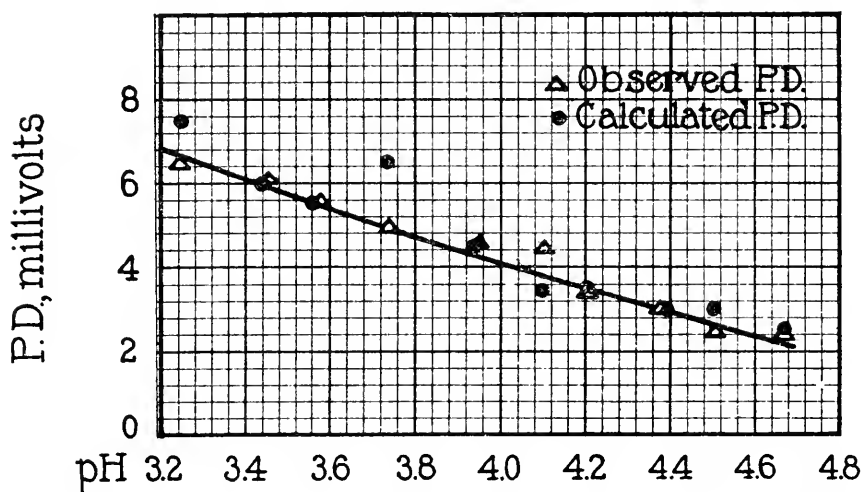


FIG. 6. Effect of pH on P. D. observed and calculated for 1 per cent edestin acetate in M/100 sodium acetate.

and calculated values for the P.D. constitute a striking proof of the validity of Donnan's theory. This experiment, therefore, supplements the results in Tables VI and VII by proving that Donnan's theory applies to solutions of edestin in acid in the region near the isoelectric point as well as in more acid solutions.

A further consequence of Donnan's theory, as applied by Loeb to amphoteric colloids, is that the pH inside should become less than the pH outside on the alkaline side of the isoelectric point of the protein; *i.e.*, the P.D. should be opposite in sign to that found on the acid side. This was tested by working with solutions of edestin in NaOH. It

was found that the solubility of edestin in acid or alkali was less than 0.45 gm. in 100 cc. for a broad zone around the isoelectric point, from about pH 5 to pH 9. Table IX gives the results of a few measurements that were made with 0.45 per cent sodium edestinate in sodium hydroxide. Both inside and outside solutions were protected from the CO<sub>2</sub> of the air by soda-lime tubes.

These results show that the prediction of the theory was confirmed, for the pH outside is now greater than the pH inside, and the sign of both observed and calculated P.D. is opposite to that obtained with the acid solutions. Moreover, the agreement between observed and calculated P.D. is close enough to show that the Donnan theory applies quantitatively on the alkaline side of the isoelectric point of edestin.

TABLE IX.

*P.D. and Osmotic Pressure of 0.45 Per Cent Sodium Edestinate.*

pH inside.....	9.93	10.41	10.96
pH outside.....	10.12	10.55	11.05
Observed P.D., millivolts.....	-9.5	-8.5	-6.0
Calculated P.D., millivolts.....	-11.0	-8.0	-6.0
Observed osmotic pressure, mm.....	28	37	45

## IV.

*Osmotic Pressure.*

The similarity between the depressing effect of salt on the osmotic pressure and on the P.D. in the case of gelatin chloride has been pointed out by Loeb.<sup>9</sup> That the same resemblance exists in the case of edestin chloride is shown by the results in Tables II to V, and by a comparison of Fig. 7 with Fig. 4. Since the abscissæ in Fig. 7 represent equivalent concentrations of salt with respect to chloride ion, and the points obtained with the three chlorides all fall on the same curve, it is evident that it is again only the anion that is effective in depressing the osmotic pressure of a solution in which the protein ion is positive. The nature or valence of the cation seems to have no effect. These results are in contradiction to the theory that the effect of a salt on such colloidal properties of protein solutions is due to the adsorption of both ions of the salt, but are in complete accord with the results of

Loeb on gelatin. The true explanation of the effect of salts on these properties of protein solutions is found in Donnan's theory of membrane equilibrium.

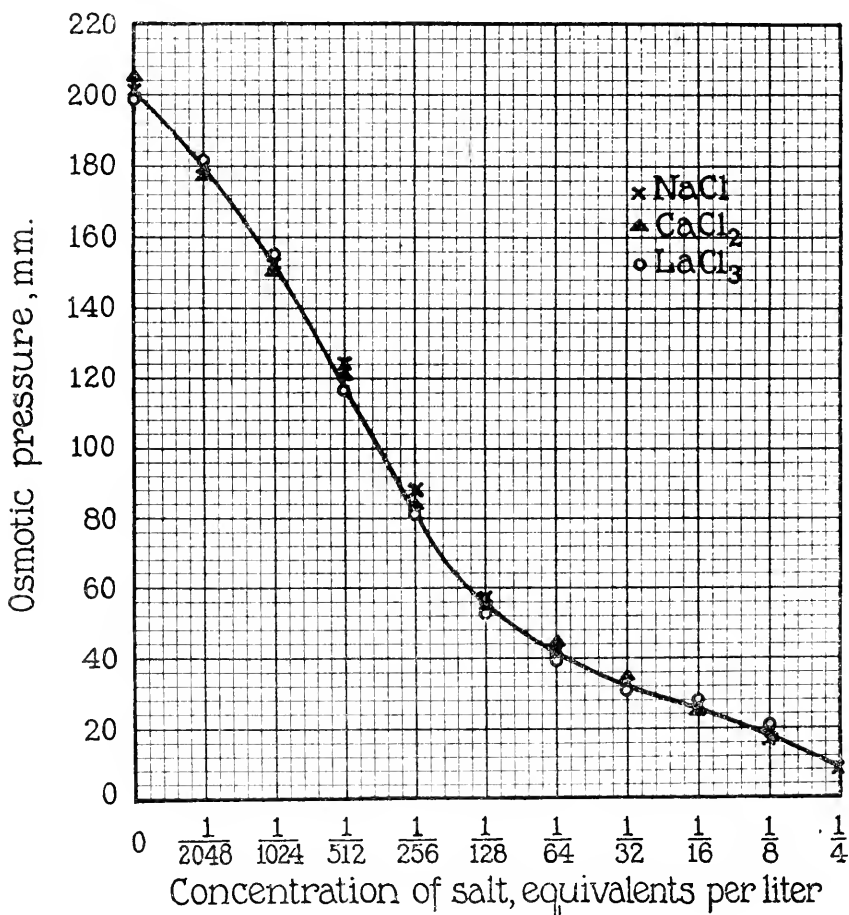


FIG. 7. Effect of different chlorides on osmotic pressure observed with 0.45 per cent edestin chloride at pH 3.

In studying the effect of hydrogen ion concentration on the osmotic pressure of gelatin chloride, Loeb<sup>9</sup> found that the curves obtained were of the same general shape as the p.d. curves, but that the maximum for osmotic pressure was at a slightly lower pH. He later<sup>10</sup>

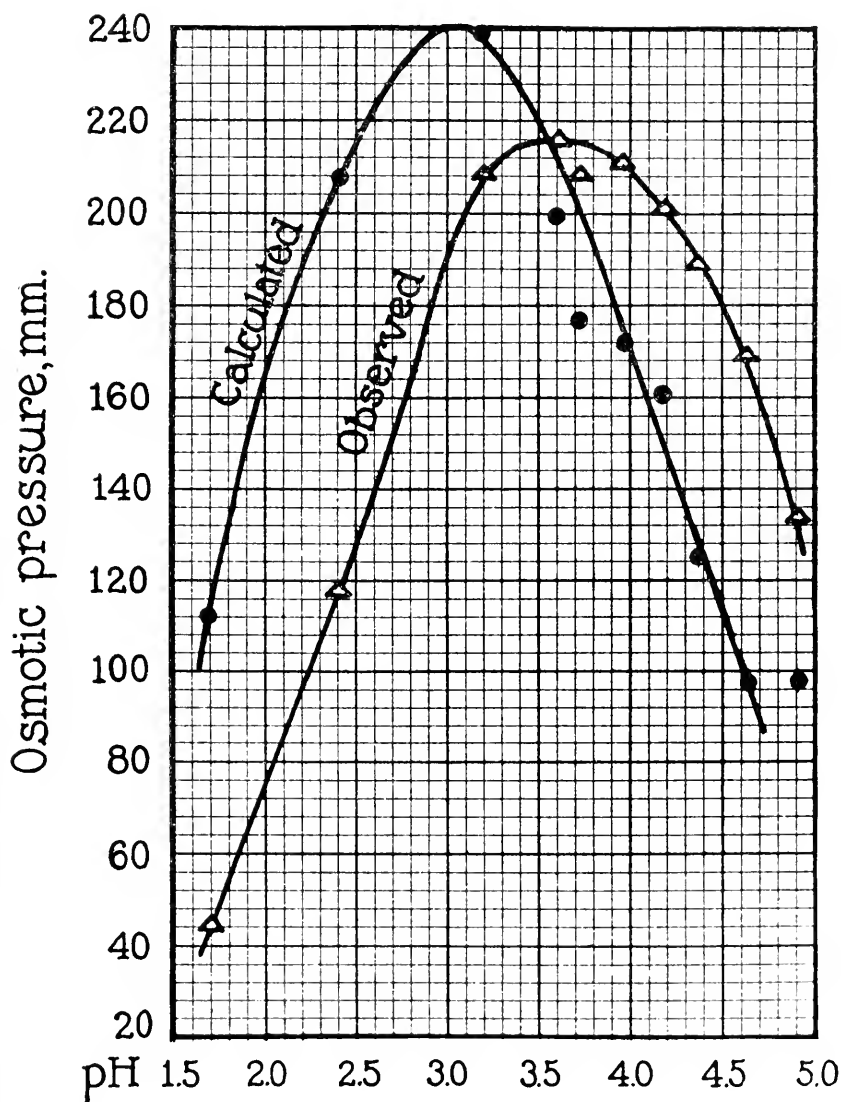


FIG. 8. Effect of pH on observed and calculated osmotic pressure of 0.45 per cent edestin chloride.

showed that the same was true for the chloride of egg albumin. On the basis of Donnan's theory he devised a method<sup>11</sup> of calculating the osmotic pressure of a protein-acid salt solution from measurements of the hydrogen ion concentration. The calculation, which neglects the unknown osmotic pressure of the protein itself, gives the following expression for the osmotic pressure in the case of a protein-acid salt with univalent anion:

$$\text{Osmotic pressure at } 24^{\circ}\text{C.} = 2.5 \times 10^5 (2y + z - 2x) \text{ mm. of water.}$$

Here  $y$  is the hydrogen ion concentration of the inside solution,  $x$  that of the outside solution, and  $z$  the concentration of anion from the protein-acid salt, all being expressed in moles per liter. Since according to Donnan's theory

$$x^2 = y(y + z)$$

the expression for osmotic pressure reduces to

$$2.5 \times 10^5 \times \frac{(x - y)^2}{y}$$

In applying this calculation to gelatin chloride, Loeb found that the curves representing osmotic pressure as a function of pH were of the same general shape and height as the observed curves, but had maxima at a lower pH. A calculation made from his results on albumin chloride<sup>10</sup> yielded similar results.

Fig. 8 represents the effect of pH on the observed and calculated osmotic pressure of a 0.45 per cent solution of edestin chloride. The curve for observed osmotic pressure has a maximum at a lower pH than that for observed P.D. shown in Fig. 5, while the curve for calculated osmotic pressure has a maximum at a still lower pH. Thus the behavior of edestin in these respects exhibits the same peculiarities which Loeb observed with gelatin and egg albumin.

#### SUMMARY.

1. It has been shown by titration experiments that the globulin edestin behaves like an amphoteric electrolyte, reacting stoichiometrically with acids and bases.

<sup>11</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 691.



2. The potential difference developed between a solution of edestin chloride or acetate separated by a collodion membrane from an acid solution free from protein was found to be influenced by salt concentration and hydrogen ion concentration in the way predicted by Donnan's theory of membrane equilibrium.

3. The osmotic pressure of such edestin-acid salt solutions was found to be influenced by salt concentration and by hydrogen ion concentration in the same way as is the potential difference.

4. The colloidal behavior of edestin is thus completely analogous to that observed by Loeb with gelatin, casein, and egg albumin, and may be explained by Loeb's theory of colloidal behavior, which is based on the idea that proteins react stoichiometrically as amphoteric electrolytes and on Donnan's theory of membrane equilibrium.

The writer wishes to acknowledge his indebtedness to Dr. Jacques Loeb, at whose suggestion and under whose direction this work was done, and to Dr. John H. Northrop, whose advice has been of great assistance.



THE ELIMINATION OF DISCREPANCIES BETWEEN  
OBSERVED AND CALCULATED P.D. OF PRO-  
TEIN SOLUTIONS NEAR THE ISOELEC-  
TRIC POINT WITH THE AID OF  
BUFFER SOLUTIONS.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 30, 1922.)

The writer has shown in a series of papers<sup>1</sup> that the P.D. observed between a solution of gelatin chloride or albumin chloride inside a collodion bag and an outside solution free from gelatin could be calculated from the difference in the hydrogen ion concentration between the inside and outside solutions. The agreement between the observed and calculated values was perfect when the solution contained a neutral salt or when the hydrogen ion concentration of the solution was not too close to that of the isoelectric point; the agreement was, however, less satisfactory when the pH was near that of the isoelectric point of gelatin, *i.e.*, near pH 4.7, and no salts were present.<sup>2</sup> The source of this disagreement seemed to lie in the inaccuracy in the measurement of the pH of the aqueous solution free from gelatin (the outside solution) at a pH between 4.0 and 7.0. If this surmise was correct, the disagreement in that region of hydrogen ion concentrations should be caused to disappear by the use of a buffer solution inside and outside.

1 per cent solutions of isoelectric gelatin were made up in M/100 Na acetate solutions containing varying amounts of 1 M acetic acid so that the pH of the gelatin solution varied (at the end of the experiment) between 4.65 (*i.e.*, practically isoelectric gelatin) and 3.34 (Table I.) Collodion bags, of a content of about 50 cc., were filled

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 667; 1921-22, iv, 351.

<sup>2</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 138, 156.

with these solutions of gelatin in buffer solutions as described in previous papers. The bags were put into beakers containing 350 cc. of identical solutions of M/100 Na acetate and 1 M acetic acid as those inside the bags, except that the 350 cc. outside solutions contained no gelatin. The temperature was 24°C. After 24 hours the osmotic pressure, the P.D. between inside and outside solutions, and the pH of the inside and outside solutions were measured. From the value pH inside minus pH outside the P.D. was calculated, and Table I shows that the P.D. thus calculated agrees with the observed P.D. The rest of the table needs no explanation.

TABLE I.

*Influence of pH on P.D. of Solutions of Gelatin Acetate in the Presence of Buffer Solution.*

Cc. 1 M acetic acid in 100 cc. inside and outside solutions.	1.0	1.5	2.0	3.0	4.0	6.0	10.0	15.0	20.0	30.0
Osmotic pressure, in mm. H <sub>2</sub> O.....	21	31	34	43	47	62	83	95	103	108
pH inside.....	4.65	4.52	4.40	4.23	4.14	3.99	3.76	3.61	3.49	3.34
pH outside.....	4.65	4.50	4.37	4.19	4.09	3.92	3.69	3.53	3.39	3.23
pH inside minus pH outside.....	0	0.02	0.03	0.04	0.05	0.07	0.07	0.08	0.10	0.11
P.D. calculated, millivolts.....	0	1.0	2.0	2.5	3.0	4.0	4.0	5.0	5.5	7.0
P.D. observed, millivolts.....	0.5	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.5	6.0

Similar results were obtained, in the case of solutions of edestin, by Dr. Hitchcock, whose paper appears simultaneously with this note.<sup>3</sup>

#### SUMMARY AND CONCLUSIONS.

1. It had been noticed in the previous experiments on the influence of the hydrogen ion concentration on the P.D. between protein solutions inside a collodion bag and aqueous solutions free from protein that the agreement between the observed values and the values calculated on the basis of Donnan's theory was not satisfactory near the

<sup>3</sup> Hitchcock, D. I., *J. Gen. Physiol.*, 1921-22, iv, 597.

isoelectric point of the protein solution. It was suspected that this was due to the uncertainty in the measurements of the pH of the outside aqueous solution near the isoelectric point. This turned out to be correct, since it is shown in this paper that the discrepancy disappears when both the inside and outside solutions contain a buffer salt.

2. This removes the last discrepancy between the observed P.D. and the P. D. calculated on the basis of Donnan's theory of P.D. between membrane equilibria, so that we can state that the P.D. between protein solutions inside collodion bags and outside aqueous solutions free from protein can be calculated from differences in the hydrogen ion concentration on the opposite sides of the membrane, in agreement with Donnan's formula.



# ELECTRICAL CHARGES OF COLLOIDAL PARTICLES AND ANOMALOUS OSMOSIS.

## II. INFLUENCE OF THE RADIUS OF THE ION.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 21, 1922.)

The valency and sign of charge of ions are not the only variables influencing anomalous osmosis. A third variable is the radius of the ion. The radius of the ions of the alkali metals increases in the order of  $\text{Li} < \text{Na} < \text{K}$ . The writer has shown that when solutions of pH 3.0 of the chlorides or nitrates of these cations are separated from a solution of water also of pH 3.0 by collodion-gelatin membranes, water diffuses into the salt solution with a rate increasing inversely with the radius of the cation.<sup>1</sup> This is illustrated in the transport curves in Fig. 1 where the abscissæ are the concentration of the salt and the ordinates the number of millimeters to which the level of liquid has risen in 20 minutes in the manometer connected with the solution. It is obvious that the rise is greatest for LiCl, less for NaCl, and still less for KCl. This confirms the results of a preceding publication.

It is also obvious that the three transport curves in Fig. 1 show the initial rise to a maximum at about  $M/16$  followed by a drop which is followed by a second rise. This second rise will not interest us here since it is mainly or exclusively the expression of the transport of liquid due to osmotic forces.<sup>2</sup> Only part of the curves, namely, between the concentration from 0 to a concentration of  $M/4$ , is due to electrical forces, and only these forces interest us in this connection.

The question arises, What determines this influence of the radius of the monatomic and monovalent cations on the electrical transport

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 673.

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 173; 1921-22, iv, 463.

of water? The idea prevalent in work on cataphoresis, electrical endosmose, or current potentials seems to be that the charge of the particle or membrane is due to the adsorption of the ions of the salt. Thus the transport curves in Fig. 1 would suggest that the cations

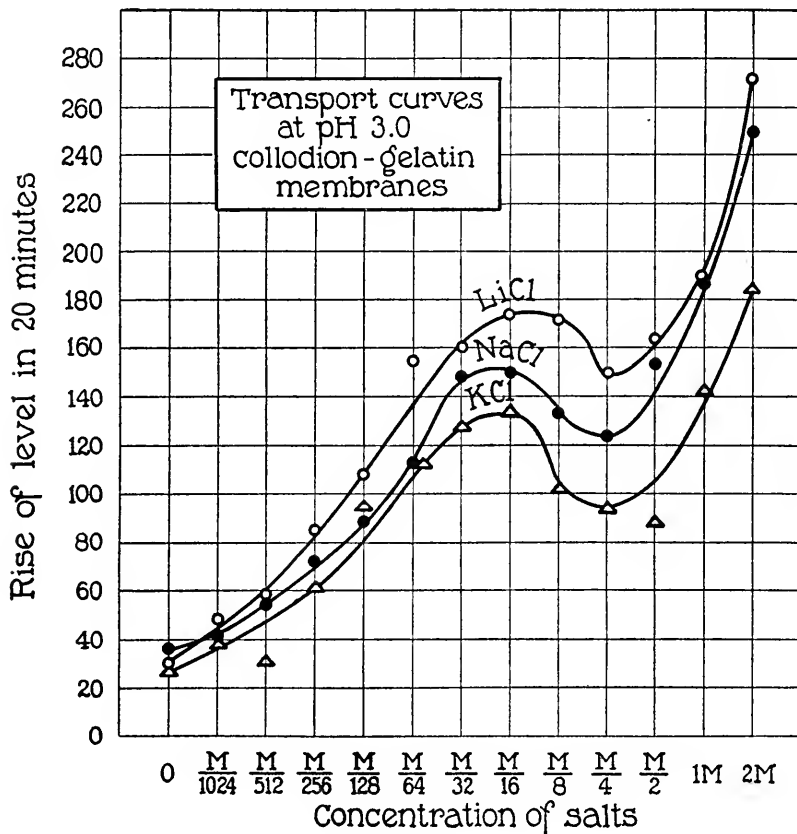


FIG. 1. Influence of Li, Na, and K on rate of electrical transport of liquid through a collodion-gelatin membrane at pH 3.0. The rate of transport increases inversely with the radius of the cation.

increase the positive charge of the walls of the pores in the gelatin membrane and that this increase occurs inversely with the radius of the three cations.

The influence of the three cations on the charge of gelatin particles was measured directly in the following way. Doses of 1 gm. of pow-



dered gelatin particles of a definite size (going through a sieve with mesh No. 30 but not through mesh No. 60) were rendered isoelectric and then put into 200 cc. of various concentrations of KCl, LiCl, or NaCl, made up in water containing 16 cc. of 0.1 N HCl at 20°C. After 2 hours, during which the mixtures were stirred frequently, the gelatin was separated from the supernatant liquid by filtration, and after this the gelatin was melted and poured into special glass vessels,

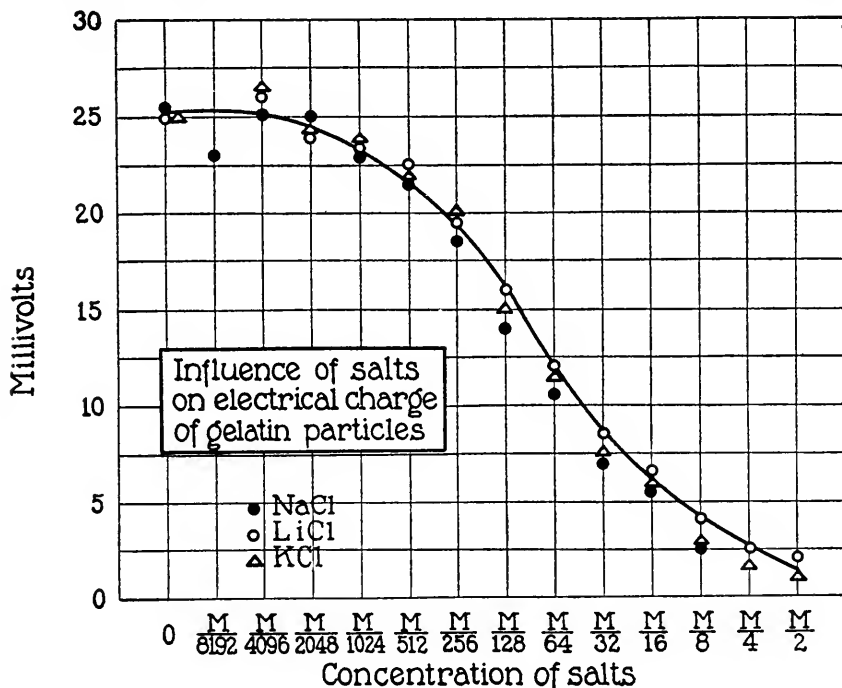


FIG. 2. Influence of LiCl, NaCl, and KCl on P.D. between solid particles of gelatin chloride and surrounding liquid at pH 3.0. This influence is the same for all three salts, suggesting that there exists only a depressing influence of the anion but no opposite influence of the cation on this P.D. Abscissæ are the concentration of the salts; ordinates, observed P.D.

and allowed to solidify in the vessels standing on ice for 1 hour. The supernatant solution was also cooled in the same way. The P.D. between the solid gelatin and the supernatant liquid with which it was in equilibrium was then measured at a temperature of about 5°C. with calomel electrodes and saturated solutions of KCl by a Compton

electrometer. The details of the procedure can be found in a book which is about to appear.<sup>3</sup>

Fig. 2 shows that the three salts, KCl, NaCl, and LiCl depress the P.D. between solid gelatin chloride and the liquid with which the gelatin is in equilibrium in exactly the same way, since the values expressing the effect of the three salts on the P.D. lie on the same curve (Fig. 2). The ordinates of these curves in Fig. 2 are the observed

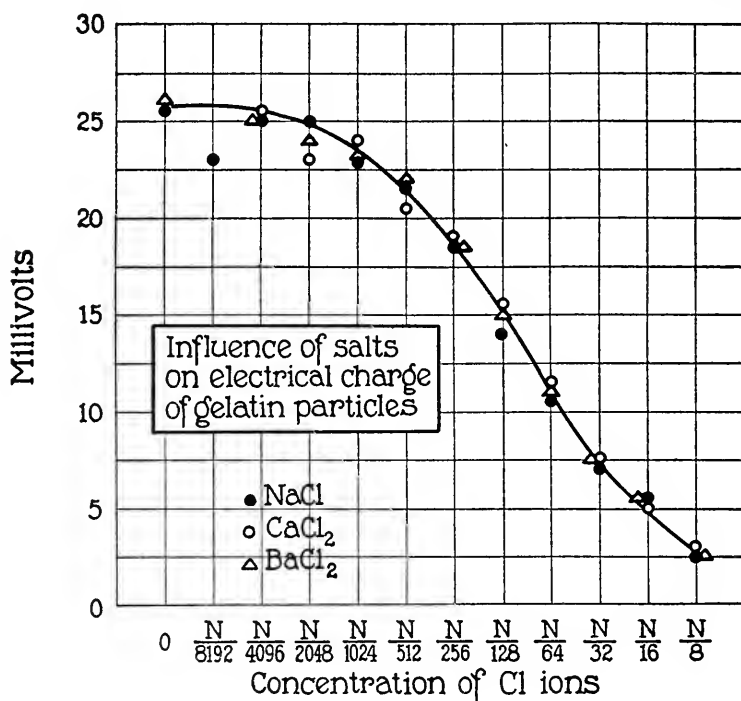


FIG. 3. Influence of NaCl, CaCl<sub>2</sub>, and BaCl<sub>2</sub> on P.D. between solid granules of gelatin chloride and surrounding liquid at pH 3.0. Abscissæ are the concentration of Cl; ordinates, observed P.D. The influence is the same for the three salts, proving that there exists only a depressing effect of the Cl ion but no opposite effect of the cation.

P.D. and the abscissæ the concentrations of the salt. These curves contradict the idea that the three cations, Li, Na, and K influence the P.D. between gelatin and water by adsorption, and, moreover, they

<sup>3</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922.

contradict the idea that the P.D. increases inversely with the radius of the three cations.

It has already been stated in the preceding paper<sup>4</sup> that at pH 3.0 only the anion of the salt has any effect on the P.D. between gelatin chloride and the liquid with which it is in contact and that this effect is only depressing. The cation of the salt has no effect. This is shown in Fig. 3, where the influence of NaCl, CaCl<sub>2</sub>, and BaCl<sub>2</sub> on the P.D. between solid gelatin chloride and the liquid with which it is in equilibrium are plotted. The method of the experiments was the same as in the experiments with LiCl and NaCl just described. The abscissæ in Fig. 3 are the chlorine ion concentrations (on the assumption of complete ionization) and the ordinates are the P.D. The influence of NaCl, CaCl<sub>2</sub>, and BaCl<sub>2</sub> on the P.D. is, therefore, the same for the same concentration of chlorine ions, which means that there exists at pH 3.0 only a depressing effect of the Cl ions on the P.D., but no increase of the P.D. through adsorption of cations. If this latter effect existed the curves in Figs. 2 and 3 representing the influence of salts on the P.D. should not be identical. The identity of the curves in Figs. 2 and 3 can only mean that that ion of a salt which has the same sign of charge as the protein ion has no effect on the P.D. between the particles of gelatin chloride of pH 3.0 and the liquid with which they are in equilibrium. It may be stated incidentally that this was to be expected if the Donnan equilibrium is the cause of the P.D.

If we now return to the interpretation of the influence of the radius of the cation on the transport curves in anomalous osmosis in Fig. 1, we must infer that the difference in the transport curves for KCl, NaCl, and LiCl is not due to any influence of the three cations on the electrical double layer inside the pores of the membrane. We have seen in the preceding paper that there exists a second P.D. which has an influence on the transport curves, namely, the P.D. across the membrane, which is essentially but not exclusively due to a difference in the mobility of the oppositely charged ions. This P.D. was measured at the beginning of each transport experiment and at the end, *i.e.*, after 20 minutes. By that time some of the salt had diffused from the salt solution into the surrounding water. Table I gives the P.D. across the membrane

<sup>4</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 463.

at the beginning and Table II gives it at the end of the experiment. The salt solution was positively charged and the outside solution was negatively charged. The reader will notice that as soon as the concentration is above  $M/64$  the P.D. across the membrane increases in the order of  $KCl < NaCl < LiCl$ , and this corresponds to the difference in the transport curves in Fig. 1 which also begins to become

TABLE I.

*Influence of Concentration of Salt on the Value of E.*

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against  $H_2O$  of pH 3.0 (acid used,  $HCl$ ), *at beginning of experiment*. Sign of charge of salt solutions positive.

Concentration.	$M/1,024$	$M/512$	$M/256$	$M/128$	$M/64$	$M/32$	$M/16$	$M/8$	$M/4$	$M/2$	1 M	2 M
	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts
KCl.....	5.0	5.0	6.5	7.5	8.0	7.5	7.5	5.5	4.5	3.5	3.5	3.0
NaCl.....	8.0	12.0	13.0	18.0	22.0	20.0	23.0	23.0	24.0	25.0	28.0	28.0
LiCl.....	6.5	9.0	12.5	18.0	21.0	25.5	27.0	35.0	37.0	37.0	40.0	42.5

TABLE II.

*Influence of Concentration of Salt on the Value of E.*

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against  $H_2O$  of pH 3.0 (acid used,  $HCl$ ), *after 20 minutes from beginning of experiment*. Sign of charge of salt solutions positive.

Concentration.	$M/1,024$	$M/512$	$M/256$	$M/128$	$M/64$	$M/32$	$M/16$	$M/8$	$M/4$	$M/2$	1 M	2 M
	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts	milli-volts
KCl.....	4.0	4.0	6.0	6.0	6.5	6.0	5.0	3.5	2.5	1.5	1.5	0.5
NaCl.....	7.5	12.0	10.0	13.0	16.5	16.0	16.0	15.0	11.0	12.0	10.5	8.0
LiCl.....	6.5	10.0	13.0	18.0	19.0	24.0	22.0	25.0	22.0	19.5	17.0	15.0

marked when the concentration of the salts rises above  $M/64$ . We must, therefore, conclude that the difference in the transport curves in Fig. 1 is due to the difference in the influence of  $KCl$ ,  $NaCl$ , and  $LiCl$  on the P.D. across the membrane. This P.D. is, perhaps, essentially, but not exclusively, a diffusion potential. Since K has the greatest and Li the smallest mobility of the three ions, it is to be expected that the diffusion potentials lead to such differences in the

P.D. as are expressed in Tables I and II. This fact has already been discussed, but it may be necessary to return to it in a later publication.

#### SUMMARY AND CONCLUSIONS.

1. When solutions of KCl, NaCl, or LiCl are separated from water without salt by a collodion-gelatin membrane and when the pH of both salt solution and water are on the acid side of the isoelectric point of gelatin, water diffuses from the side of pure water into the salt solution at a rate increasing inversely with the radius of the cations.

2. The adsorption theory would lead us to assume that this influence of the cations is due to an increase of the P.D. between the liquid and the membrane inside the pores of the gelatin film of the membrane, but direct measurements of this P.D. contradict such an assumption, since they show that the influence of the three salts on this P.D. is identical at pH 3.0.

3. It is found, however, that the P.D. across the membrane is affected in a similar way by the three cations as is the transport of water through the membrane.

4. This P.D. across the membrane varies inversely as the relative mobility of the three cations which suggests that the influence of the three cations on the diffusion of liquid through the membrane is partly if not essentially due to a diffusion potential.



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JACQUES LOEB

W. J. V. OSTERHOUT

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## THE STABILITY OF BACTERIAL SUSPENSIONS.

### I. A CONVENIENT CELL FOR MICROSCOPIC CATAPHORESIS EXPERIMENTS.

BY JOHN H. NORTHROP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 10, 1922.)

Measurements of cataphoresis of particles in an electric field are complicated by the fact that in addition to the motion of the particles relative to the water, the water itself moves at the surface of the cell. In macroscopic measurements the cell is large enough so that the movement of the water as a whole is negligible. If the measurements are made in a narrow cell, however, the motion of the water is very great and must be corrected for. The theory and technique of such measurements have been thoroughly discussed by Ellis<sup>1</sup> and Powis.<sup>2</sup> As Ellis pointed out, the total motion of the water in a closed cell must be zero, since the water which moves one way at the surface of the glass must return in the opposite direction in the center of the cell. The *average* observed motion of the particles at all depths relative to the cell must, therefore, be the true motion of the particles relative to the water, which is the desired value. It is also necessary to use some form of non-polarizable electrodes in order to avoid the formation of gas bubbles. The cell devised by Powis answers the requirements but is troublesome to use if a large number of experiments are made. The cell shown in Fig. 1 has been found very convenient.

*Construction of the Cell.*—The cell itself is made of a thin slide resting on strips of glass about 0.8 mm. thick cemented to a thick glass slide. Two blocks of thick glass are cemented on top of the cover-slide at each end of the cell. The ends of the cell are then ground smooth on an emery wheel. A piece of thick walled glass tubing is widened and flattened at one end so as to cover the end opening of the

<sup>1</sup> Ellis, R., *Z. physik. Chem.*, 1911, lxxviii, 321; 1912, lxxx, 597.

<sup>2</sup> Powis, F., *Z. physik. Chem.*, 1914, lxxxix, 91.

cell. This flattened end is ground smooth and is cemented to the end of the cell. The same process is repeated at the other end of the cell. The method of arranging the electrodes is apparent from the figure. The best material for cementing the cell together was found to be soft "De Khotinsky cement." The pieces of glass are warmed to about  $80^{\circ}$  in an air bath, coated with a thin layer of cement and pressed together. The surplus cement is then removed with wire and the last traces are wiped off with a piece of paper moistened with toluene. After the end-pieces have been fastened to the cell it is

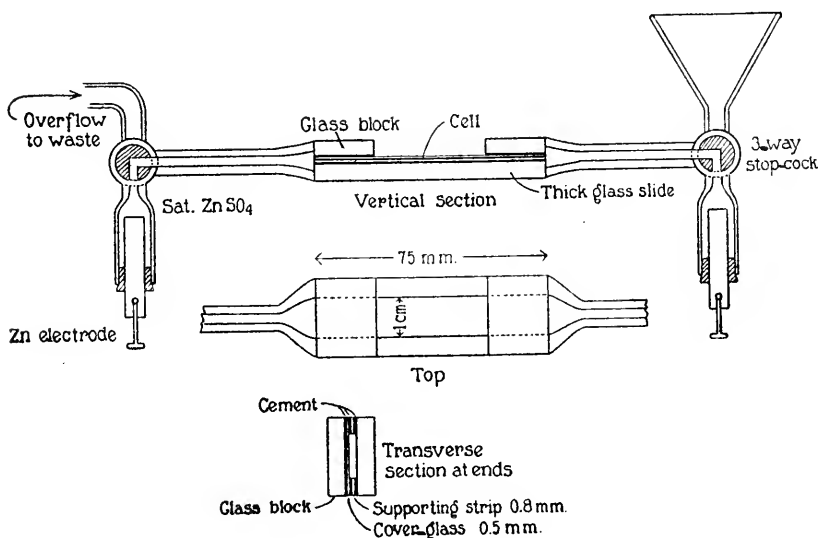


FIG. 1.

advisable to run more cement around the joint to strengthen it; this can easily be done by using a small soldering iron, the cement being handled in the same way as solder.

*Calibration of the Cell.*—It is necessary to know the drop in potential per cm. in the cell itself. Since the area of the connecting tubes, etc. in general is different from that of the cell it is necessary to correct for this difference. If the apparatus as a whole is filled with the same solution, as is the case during an experiment, the total resistance of the solution will evidently be proportional to the length and inversely proportional to the cross-section. Since the drop in potential per cm.

is proportional to the resistance per cm., it is possible to calculate the drop in potential in the cell itself, provided the dimensions of the rest of the apparatus are known. Expressed as an equation,

$$\text{Drop in potential per cm. in the cell} = \frac{\frac{L_c}{A_c}}{\frac{L_c}{A_c} + \frac{L_t}{A_t} + \frac{L_{t'}}{A_{t'}}} \times \text{total drop in potential}$$

$L_c$  = length of cell in cm.

$A_c$  = area of cross-section of cell.

$L_t$  = length of tubing in cm.

$A_t$  = area of cross-section of tubing.

The total potential is measured by a voltmeter connected to the Zn. electrodes.

In experiments with salt concentrations of less than tenth normal the resistance of the zinc sulfate is negligible. In any case the uncertainties due to the change in dielectric constant with increasing salt concentration are probably greater than the error introduced by neglecting the zinc sulfate. It can be seen from the above that failure to allow for a widening of the system at some point would make little or no difference in the potential gradient but that a narrow place in the system even though very short would cause a very large error. It is important, therefore, to be sure that the cell is not narrowed at the ends by the cement or by failure to align the ends of the cell and the side tubes.

*Method of Measuring the Velocity of Migration.*—The cell is clamped in position under the microscope, after filling the electrode tubes with saturated zinc sulfate, and the electrodes connected to a source of potential. The stop-cocks are turned so as to close the tubes containing the zinc sulfate and the cell filled with the suspension, care being taken to avoid air bubbles. The stop-cocks are then turned so as to connect them with the zinc sulfate solution, the circuit closed and the time required for a particle to cross a division of the micrometer eye-piece determined with a stop-watch. Owing to the migration of the water itself, it is necessary to obtain the average motion of the particles in the cell as a whole. This may be done accurately by determining the speed at various depths, say every 0.05 mm., plotting the curve so obtained and determining the mean height from the area

as measured by a planimeter and the length of the base. This is a time-consuming procedure and Ellis proposed a correction formula which required only two measurements, one at the surface of the cell and one at the center. This formula however, is based on the assumption that the velocity of the water in the center of the cell bears a constant relation to the velocity of the water at the walls of the cell. This ratio in turn depends upon the viscosity of the solution. The writer found that in the presence of proteins, serum, etc., the formula did not hold, owing presumably to changes in viscosity. It was found, however, that a value could be obtained which agreed with the true mean value by making measurements at the middle of each sixth or each eighth of the cell. Since the upper and lower halves of the cell are symmetrical, this requires either three or four measurements. It was found that the results were more reliable if a few measurements were taken at four depths than if the same total number of measurements were made at three depths. The procedure adopted was as follows: The apparent depth (on account of diffraction this is three-fourths of the actual depth) of the cell was 0.64 mm. as measured by the micrometer screw of the fine adjustment. The velocity of the particles was therefore measured at a distance of 0.04, 0.12, 0.20, and 0.28 mm. from the top of the cell, corresponding to the center of the first four eighths of the cell. Four measurements were made at each depth and the average of the reciprocals of these values taken as the true average velocity of the particles relative to the water. It is advisable to have a reversing switch in the circuit and take alternate measurements with the current reversed. These measurements should agree, and any divergence can usually be traced to air bubbles or a leak in the cell. When there is no potential across the cell the particles should remain stationary. Table I is an example of an experiment.

The potential is calculated from the observed velocity by means of the Helmholtz-Lamb equation as discussed in the preceding paper.<sup>3</sup>

*Accuracy of the Method.*—It was found in general that the measurements could be repeated within 1 to 2 millivolts. The calculated error from one series of measurements is considerably less than that, but the difference is probably due to the difficulty of making the measurements at exactly the correct depth. It is necessary, of course, to count

<sup>3</sup> Northrop, J. H., and Cullen, G. E., *J. Gen. Physiol.*, 1921-22 iv, 635.

only those particles that are sharply in focus. This error could be lessened by using a deeper cell. It was found, however, that in a cell 2 mm. deep there was irregular drifting of the particles. Since the final value depends as a rule on the difference of two experimental values, the percentage error is larger the smaller the velocity.

TABLE I.  
*Rate of Migration of B. typhosus Suspension in Distilled Water.*  
Potential gradient = 4.5 volts per cm.

Distance from cover-glass at which determination was made.	Time to go 45 $\mu$ .	Average time.	Average $\mu$ per second.
<i>mm.</i>	<i>sec.</i>	<i>sec.</i>	$\mu$
0.04	+15.0 +16.0* +15.5 +14.0	+15.2	+2.95
0.12	-4.5 -5.0 -4.0 -5.0	-4.6	-9.8
0.20	-2.5 -2.3 -2.4 -2.6	-2.45	-18.2
0.28	-2.0 -1.8 -2.0 -2.0	-2.0	-22.5
Average $\mu$ per second at all depths.....			-11.9
Average $\mu$ per second calculated for potential gradient of 1 volt per cm.			-2.65

Potential bacteria water, -33.5 millivolts.

\* The sign refers to the apparent sign of charge of the particle, *i.e.* + indicates migration to the cathode.

*Apparent Reversal of the Charge on the Glass.*—It was noted that the direction of migration of the water reversed at times under the same conditions that caused a reversal in the motion of the particles. It is known that the sign of the charge on glass cannot be reversed so that this result is at first sight anomalous. The explanation is simple, however, since microscopic examination of the cell shows that it becomes more or less coated with the organisms, which adhere firmly to the glass. The cell wall is, therefore, no longer glass but is partially composed of the same material as the suspension and therefore reverses its charge under the same conditions as does the suspension.





## AN APPARATUS FOR MACROSCOPIC CATAPHORESIS EXPERIMENTS.

By JOHN H. NORTHROP AND GLENN E. CULLEN.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

(Received for publication, May 10, 1922.)

The form of apparatus<sup>1</sup> shown in Fig. 1 has been found very convenient for the determination of the migration of fine suspensions in the electric field. Its essential difference from the usual cataphoresis apparatus is that the whole is inverted so that the central portion, which contains the suspension which is to be studied, is above the heavier electrode solutions and may be left open. This increases both the convenience and accuracy of method since (1) a greater latitude in concentration of solution is allowed, (2) the boundaries may be adjusted more exactly, and (3) the solution may be renewed without disturbing the electrode solution. The zinc electrodes are put in place with rubber stoppers, the tube is clamped in a vertical position, and the apparatus is filled with saturated zinc sulfate. The three-way stop-cocks are then closed and the zinc sulfate in the upper part of the cell is washed out through the "tail holes." The tubes above the stop-cocks are now filled with 0.1 M sucrose solution containing the same concentration of electrolyte or other substance as is to be used with the suspension. The sugar solution is then allowed to run out until the level reaches the small tube connecting the funnel and the U-tube. The suspension is then added and the level adjusted carefully by means of the stop-cocks so that the line of demarcation is opposite one of the graduations on the side arms. The upper stop-cock is then closed and the lower ones are opened so as to connect the zinc sulfate with the sugar solutions. The current is applied and the distance traversed by the boundary determined after a convenient interval.

<sup>1</sup>For a discussion of this and similar methods see Burton, E. F., *The physical properties of colloidal solutions*, London, New York, Bombay, Calcutta, and Madras, 2nd edition, 1921.

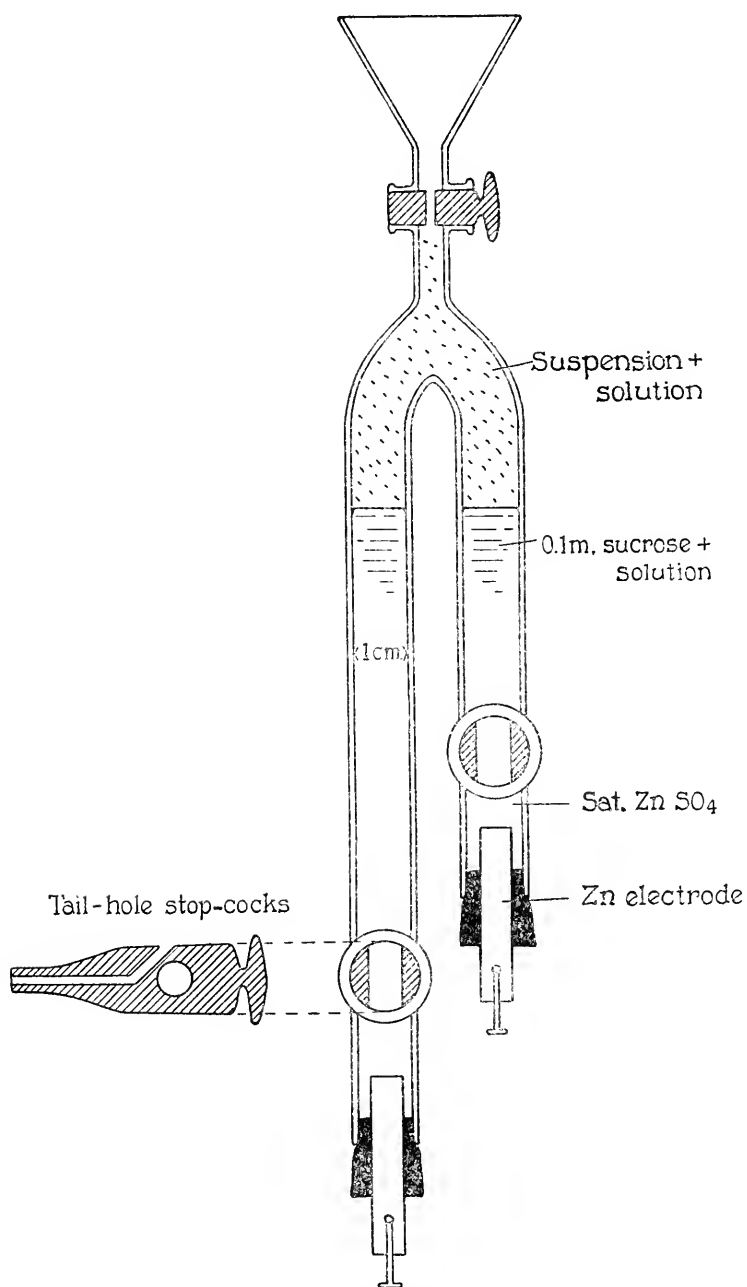


FIG. 1.

Since the cell is of uniform diameter the drop in potential is determined simply by dividing the total voltage by the distance between the three-way stop-cocks. (In solutions of less than 0.1 *N* the drop in potential in the saturated zinc sulfate may be considered as negligible; if more concentrated solutions are used it is necessary to apply a small correction for the resistance of the zinc sulfate.)

*Influence of the Voltage.*—It was found that the rate of migration was directly proportional to the voltage between the limits of 1 to 4 volts per cm., provided the experiment was not allowed to run too long. At low voltages, the rate of migration remained constant until the boundary approached the zinc sulfate, but if the potential drop was increased beyond 2 volts per cm., it was found that the boundary moved at the proper rate for the first 4 to 5 mm., but then became much too slow on one or both sides. There was also a tendency for the boundary to become convex on one side, showing that the migration of the water was interfering with the measurement. If the solution is of high conductivity, the voltage must be still further decreased to prevent heating effects and subsequent convection currents.

*Influence of the Sugar Concentration.*—The presence of sugar greatly facilitates the adjustment and maintenance of a sharp boundary line. No effect on the velocity of migration could be observed up to 0.5 *M*. Higher concentrations than this decrease the velocity presumably on account of the viscosity.

*Calculation of the Potential from the Velocity of Migration.*—The value for the potential difference between the particle and the surrounding solution is calculated by means of the Helmholtz-Lamb equation<sup>1</sup>

$$\text{P.D.} = \frac{4 n v \pi}{K X}$$

in which

*n* = viscosity of the solution.

*v* = velocity of particle in cm. per second.

*K* = dielectric constant of the solution.

*X* = potential gradient; *i.e.*, the drop in potential in E.S.U. per cm.

All electrical units are electrostatic.

Substituting for the viscosity (0.009) and dielectric constant of water at 20° (80), and converting to volts it is found that P.D. in millivolts =  $12.6 \times \mu$  per sec.  $\times$  volts per cm. =  $4.5 \times$  mm. per hour  $\times$  volts per cm.

In the experiments to be described no correction was made for a change in the dielectric constant nor viscosity.

The distance is taken as the average of the observed movement in the two arms. This corrects for any gravity effect. As a rule, however, the two readings are nearly identical and any marked discrepancy is apt to be the result of a leak in the apparatus or some other accidental error.

The apparatus has been found to work very satisfactorily for gelatin, edestin, the bacillus of rabbit septicemia, and other fine suspensions. With suspensions containing larger particles such as casein, typhoid bacilli, etc., the line of demarcation becomes blurred and the results are not satisfactory. For any particles that can be seen without an oil immersion lens the microscopic method is better.

*Accuracy of the Measurements.*—The measurements can usually be repeated as closely as can be read; *i.e.*,  $\pm 0.1$  mm. The error is greater in very dilute and very concentrated salt solutions.

## THE STABILITY OF BACTERIAL SUSPENSIONS.

### II. THE AGGLUTINATION OF THE BACILLUS OF RABBIT SEPTICEMIA AND OF BACILLUS TYPHOSUS BY ELECTROLYTES.

By JOHN H. NORTHROP AND PAUL H. DE KRUIF.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

(Received for publication, May 22, 1922.)

It is a very old observation that the stability of suspensions is markedly affected by the addition of electrolytes.<sup>1</sup> Under certain conditions the particles remain separate, while under other conditions they adhere to each other. In the latter case the particles settle rapidly and the suspension is said to be coagulated or agglutinated.<sup>2</sup> Since under certain conditions the particles remain distinct and in others collect into large masses, it is evident that there is a force which tends to hold them together and another force which tends to keep them apart. If the attractive force is greater than the repulsive force, the particles agglutinate. It was early found that nearly all substances in suspension are electrically charged with reference to the surrounding liquid, and it was suggested by Jevons that the repulsion due to this charge was the repelling force. This conception was substantiated by Hardy, who found that suspensions of denatured proteins coagulated at the point at which they carried no electric charge. Hardy called this the isoelectric point. Hardy's experiments have been greatly extended by Michaelis and his coworkers. It is probable, however, that the precipitation of proteins and the agglutina-

<sup>1</sup> For a review of the literature on this subject see Burton, E. F., *The physical properties of colloidal solutions*, London, New York, Bombay, Calcutta, and Madras, 2nd edition, 1921. In regard to proteins see Michaelis, L., *Die Wasserstoffionenkonzentration*, Berlin, 1914.

<sup>2</sup> The rapid settling of the coagulated suspension is a secondary phenomenon due to the increase in size of the particles and governed presumably by Stokes' law. The primary phenomenon is the adherence or repulsion of the individual particles.

tion of suspensions are governed by entirely distinct forces. In the case of oil emulsions Powis<sup>3</sup> was able to show that agglutination occurred whenever the potential became less than a certain critical value, in this case about 30 millivolts. Powis' experiments leave little doubt that the potential at the oil-water surface is the determining factor in the agglutination of oil emulsions. Burton<sup>1</sup> also found that metallic suspensions coagulate in the zone where the potential is small, although he did not find such a definite critical value.

In the case of bacteria, however, the results have been much less satisfactory. It was found by Bechhold,<sup>4</sup> Arkwright,<sup>5</sup> Teague and Buxton,<sup>6</sup> and others that bacteria were always negatively charged whether or not they were agglutinated. These authors concluded, therefore, that the potential carried by the organisms could not account for the phenomena. Putter<sup>7</sup> was able to show some qualitative agreement between the potential and agglutination of *Bacillus typhosus*.

#### *Results of the Present Experiments.*

It is evident that in order to test the hypothesis outlined above, it is necessary to measure both the force which tends to cause the particles to adhere as well as that which keeps them apart, since if both forces are affected by the conditions of the experiment but only one is measured, it will be impossible to interpret the results. The potential may be conveniently measured by the rate of migration in an electric field. The attractive forces, however, have usually been assumed to remain constant and no attempt has been made to measure them. It was found, in the course of the present experiments, that a comparative measure of the attractive forces between the organisms could be obtained by measuring the force required to tear apart two glass plates covered with a film of the bacteria and immersed in the solution which was under investigation. As a result of

<sup>3</sup> Powis, F., *Z. physik. Chem.*, 1914, lxxxix, 186.

<sup>4</sup> Bechhold, H., *Z. physik. Chem.*, 1904, xlviii, 385.

<sup>5</sup> Arkwright, J. A., *J. Hygiene*, 1914, xiv, 261.

<sup>6</sup> Teague, O., and Buxton, B. H., *Z. physik. Chem.*, 1907, lvii, 76.

<sup>7</sup> Putter, E., *Z. Immunitätsforsch., 1te Abt., Orig.*, 1921, xxxii, 538.

these measurements in conjunction with the measurements of the potential difference, it has been found that whenever the potential difference between the surface of the bacteria and the solution is less than about 15 millivolts the bacteria agglutinate, *provided the cohesive force is not affected*. If the cohesive force is decreased, this critical potential is decreased, and if the cohesive force is made very small, no agglutination occurs even though the potential be reduced to zero. It was further found that all electrolytes tested in concentrations less than 0.01 to 0.1 *N* affect primarily the potential, while in concentrations greater than 0.1 *N* the effect is principally on the cohesive force. In the case of bacteria sensitized with immune serum, the cohesive force remains constant and the agglutination can be predicted solely from the measurement of the potential.

### *Experimental Methods.*

*Measurement of the Potential.*—The potential was determined from the rate of migration as described in the preceding papers.<sup>8</sup> The U-tube method was used for the experiments with the bacillus of rabbit septicemia and the microscopic method with *B. typhosus*.

*Measurement of the Cohesive Force.*—A piece of thick glass slide was covered with a thin film of very heavy suspension of washed organisms (*B. typhosus*), the film allowed to dry and then heated to 60° for a few minutes. This causes the bacteria to adhere firmly to the glass. A heavy (No. 3) cover-slip was similarly prepared. The cover-slip was suspended by means of a fine platinum wire from the lever of the du Noüy<sup>9</sup> surface tension apparatus. The glass slide was immersed in a dish containing the solution to be studied and the cover-slip allowed to rest on it with its own weight for 1 minute. The force required to pull the cover-slip from the slide was then determined. It was found that if the measurement was made immediately after the two surfaces came in contact, the value obtained depended on the force with which the two had been pressed together. If the slip had been pressed down firmly a much greater force was required than if it had simply been allowed to rest on the slide. After a short time interval, however, this difference became less, and eventually the same reading was obtained in both cases. This is due presumably to the fact that the distance apart of the two surfaces is regulated by capillary forces and comes to the same point from either side. The same smear was used as long as the same value was obtained on replacing the preparation in distilled water. The value obtained becomes less after ten or

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<sup>8</sup> Northrop, J. H., and Cullen, G. E., *J. Gen. Physiol.*, 1921-22, iv, 635. Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 629.

<sup>9</sup> du Noüy, P. L., *J. Gen. Physiol.*, 1918-19, i, 521.

fifteen measurements due to the gradual removal of the film. Control experiments with clean glass surfaces showed no significant variation under the conditions of the experiment. The values obtained in this way were surprisingly reproducible. They have been expressed as milligrams required to separate two surfaces each 2 cm. square. The results are not exactly comparable to the measurements of the potential since the organisms have been subjected to dry heat. It will be noted, in fact, that the results do not conform exactly to those expected from the potential measurements. In the case of NaCl, for instance, the concentration required to affect the cohesive force noticeably, is slightly higher than would be expected from the potential curve.

It has usually been considered that this force is a surface tension effect, but there does not appear to be any conclusive evidence as to its nature. It is better, perhaps, to refer to it simply as "cohesive" without an exact definition of its nature.

*Measurement and Regulation of the Hydrogen Ion Concentration.*—The pH determinations were made electrometrically, using a saturated calomel cell and taking the pH value of 0.10 N HCl as 1.04 at 33° as the standard.

*Buffers Used.*—It was found that a very convenient buffer could be made by combining sodium phosphate, sodium acetate, and glycine. It may be used over a range of pH from 1 to 13 and has the further advantage that the nature of ions present is not varied. The only variation is a change in concentration of the Cl and Na ions. The composition and the titration curve of the buffer are given in Fig. 1. This is referred to as G. P. A. Buffer. The pH measurements were made at 33°. In some experiments Walpole's<sup>10</sup> acetate series was used.

*Cultures.*—The culture of the bacillus of rabbit septicemia used was that previously isolated and described by one of the writers.<sup>11</sup> The typhoid culture was the Pfeiffer strain obtained through the kindness of Dr. Charles Krumwiede to whom we are also indebted for the strong antityphoid horse serum.

*Measurement of the Degree of Agglutination.*—No satisfactory method could be found for measuring the agglutination quantitatively. Several degrees of agglutination were, therefore, selected and the determinations made on this basis. They were recorded as follows:

- No agglutination.
- + Distinct particles visible with a lens, 8 diameters magnification.
- ++ Particles visible with the eye alone.
- +++ Suspension almost completely agglutinated and settled but cloudy appearance in the supernatant liquid.

C. Supernatant liquid perfectly clear.

The stage marked C. is the easiest to detect with certainty and was used as the end-point.

The degree of agglutination increases with time at first but after 24 hours remains constant. All readings were therefore made after 24 hours at 20° to elimi-

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<sup>10</sup> Walpole, G. S., *J. Chem. Soc.*, 1914, cv, 2501.

<sup>11</sup> De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.



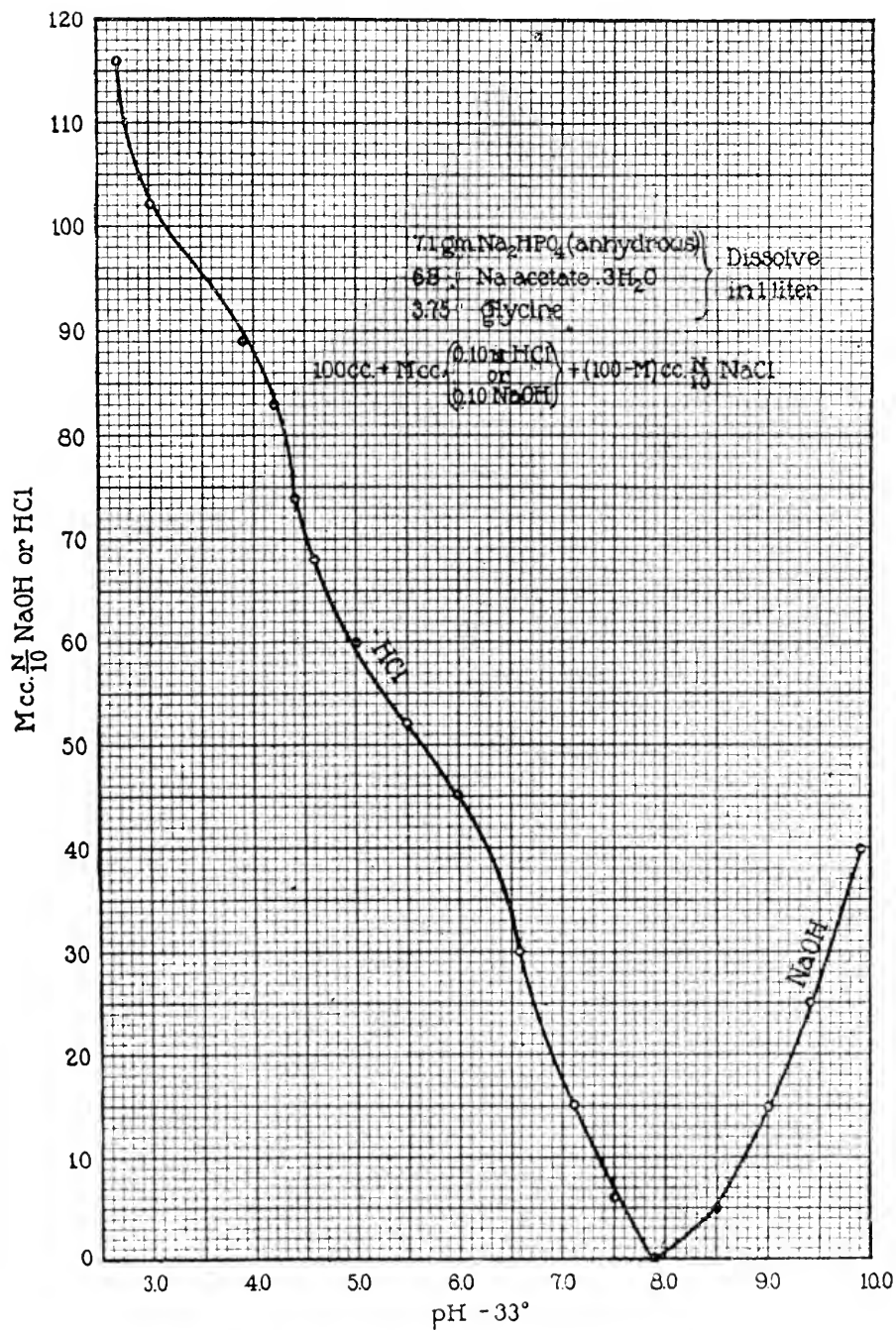


FIG. 1. Titration curve of glycine acetate phosphate buffer.

nate the time factor. A typical experiment is shown in Table I. It is evident that there is some relation between the charge and the rate of agglutination. The suspensions having the lowest charge are the ones which agglutinate the most rapidly. The table shows, however, that the relation is not continuous. Those suspensions having a potential greater than about 15 millivolts do not agglutinate completely at any time. In other words, the potential does not merely effect the time required for agglutination, but if larger than a certain value, prevents it entirely. This is the result obtained by Powis.<sup>3</sup> The fact that the point of agglutination is not sharp but covers a fairly wide range between no agglutination and complete agglutination, may be due to the individual variation in the

TABLE I.

*Effect of Potential and Time of Standing on Agglutination.*

Suspension of Type D<sup>11</sup> in acetate buffer pH 4.2 + noted concentration of egg albumin pH 4.2.

Concentration of egg albumin.	Mm. per hr.	Potential difference.	Agglutination after time noted at 20°C.				
			0.5 hr.	1 hr.	4 hrs.	24 hrs.	48 hrs.
<i>per cent</i>		<i>millivolts</i>					
0	-7.5	-34.0	—	—	—	—	—
0.0003	-6.0	-27.0	—	—	—	+	+
0.001	-4.0	-18.0	—	—	+	++	++
0.003	-2.0	-9.0	—	+	++	C.	C.
0.010	0	0	+	++	C.	C.	C.
0.03	+0.8	+3.4	—	++	++	C.	C.
0.10	2.0	+9.0	—	+	++	C.	C.
0.30	2.5	+11.2	—	—	+	C.	C.
0.90	3.2	+14.4	—	—	—	C.	C.

+ = agglutination visible with lens (8 diameters).

++ = agglutination visible without lens.

C. = complete settling, supernatant clear.

particles. It would be better theoretically, therefore, to use the point of half coagulation as the end-point. This cannot be determined experimentally owing to the lack of a quantitative method for determining the degree of agglutination.

*Preparation of the Suspension.*—It has been noted by one of us<sup>12</sup> that the presence of traces of peptones, etc., present in the culture medium, markedly affect the agglutination of the organisms. The suspensions were therefore thoroughly washed in distilled water. 24 hour broth cultures of the organisms were centrifuged, and resuspended in distilled water. This process was repeated four

<sup>12</sup> De Kruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 395. See also Putter.<sup>7</sup>

times. The sediment finally obtained was then suspended in a volume of distilled water equal to one-half that of the original broth. For the determinations one volume of this "standard" suspension was added to one or two volumes of the other solutions used. Table II shows that no noticeable change could be detected after the second washing.

*Effect of the Manner of Mixing and Time of Standing on the Potential.*—No difference could be detected in the results obtained when the suspension was added to the solution or *vice versa*, provided the mixing was rapid and complete. As a rule the suspension was squirted into the solution from a pipette and mixed as thoroughly and rapidly as possible. No significant changes occurred in the potential measurements over an interval of 2 days except in the case of silver salts. The effect on the potential is, therefore, almost instantaneous in most cases. This is also true of the effect of immune serum, and shows that the time element consists in the time required for the organisms to come into contact.<sup>13</sup> In

TABLE II.

*Effect of Washing on Rate of Migration of Type D Suspension.*

100 cc. of broth culture, Type D, were centrifuged, suspended in distilled H<sub>2</sub>O, centrifuged, and the process was repeated as noted. Migration was determined as noted.

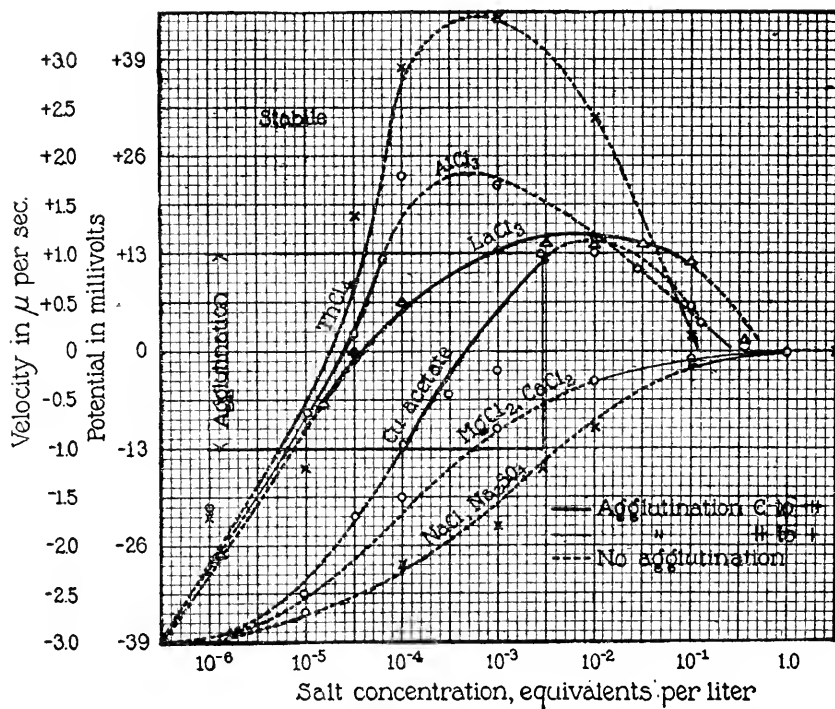
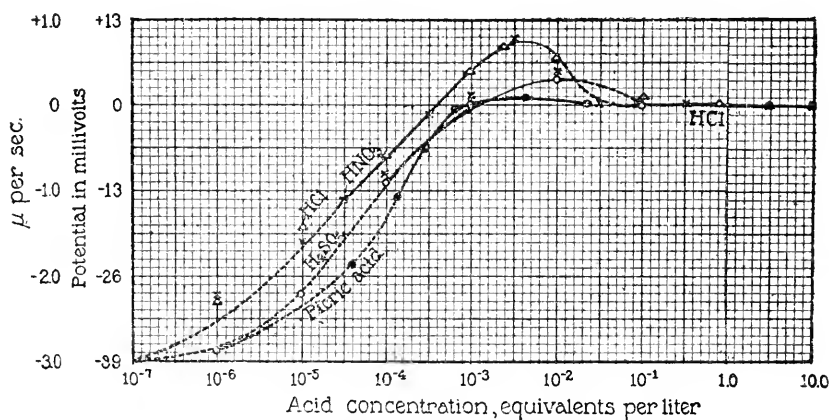
No. of times washed.....	0	1	2	3	4
Potential at pH 4.4.....	-9.0	-18.0	-27.0	-28.0	-28.0
" " pH 3.0.....	-13.0	+1.6	+1.6		+1.8

the case of suspensions treated with silver salts at a pH of 4 or more, the potential drops rapidly and is very much lower after 24 hours. At the same time the suspension turns black so that the effect is probably due to the reduction of the silver.

#### EXPERIMENTAL RESULTS.

The results of the experiments are shown graphically in Figs. 2 to 8. The calculated potential in millivolts between the surface of the organism and the surrounding liquid is plotted as ordinates, and the salt concentration as abscissae. Since there is some doubt as to the correctness of the formula connecting velocities to millivolts, the actual velocities corrected for a potential drop of 1 volt per centimeter have also been given. The degree of agglutination is indicated by the character of the line. In the experiments in which no pH value

<sup>13</sup> This conclusion had been reached by F. L. Gates (*J. Exp. Med.*, 1922, xxxv, 63) in a study of the time required for adsorption of immune body.

FIG. 2. Effect of salts on the potential and agglutination of *B. typhosus*.FIG. 3. Effect of acids on the potential and agglutination of *B. typhosus*.

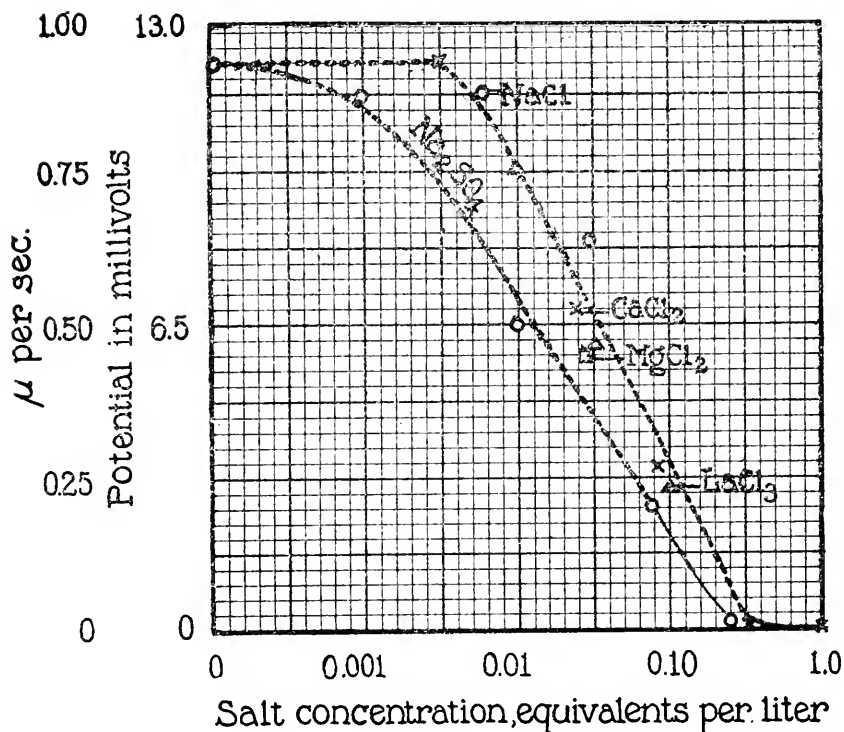


FIG. 4. Effect of salts on the potential and agglutination of *B. typhosus* at pH 2 (0.01 N HCl).

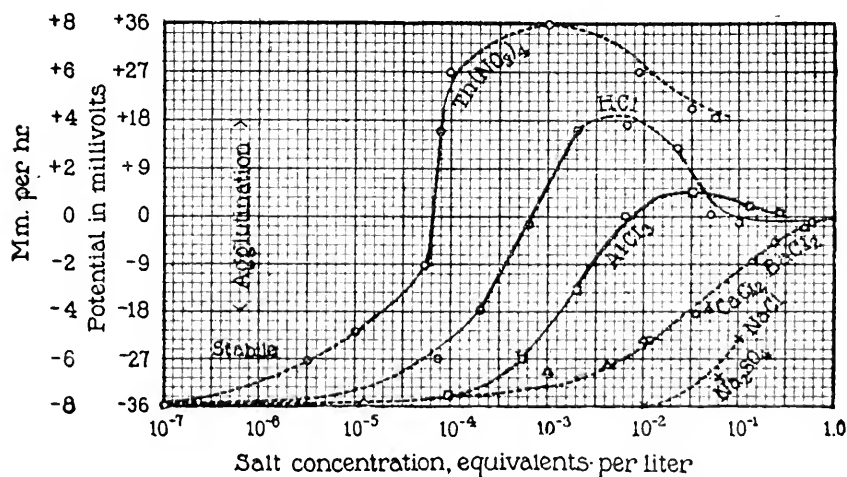


FIG. 5. Effect of salts on the potential and agglutination of the bacillus of rabbit septicemia Type D strain.

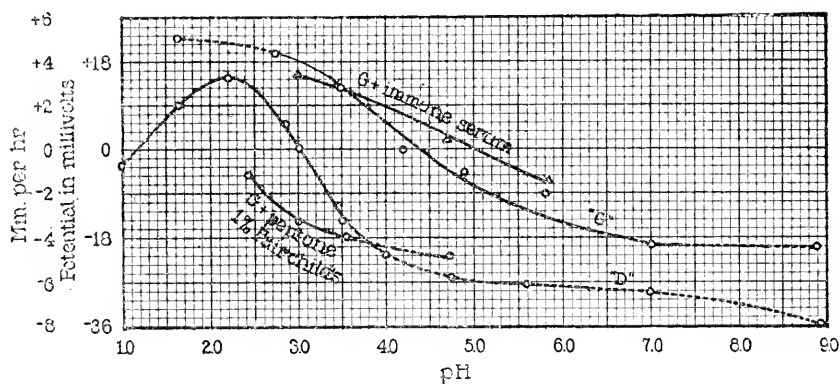


FIG. 6. Comparison of the acid agglutination of Type D and Type G strains of the bacillus of rabbit septicemia and the effect of immune serum and peptone on the potential and agglutination of Type G.

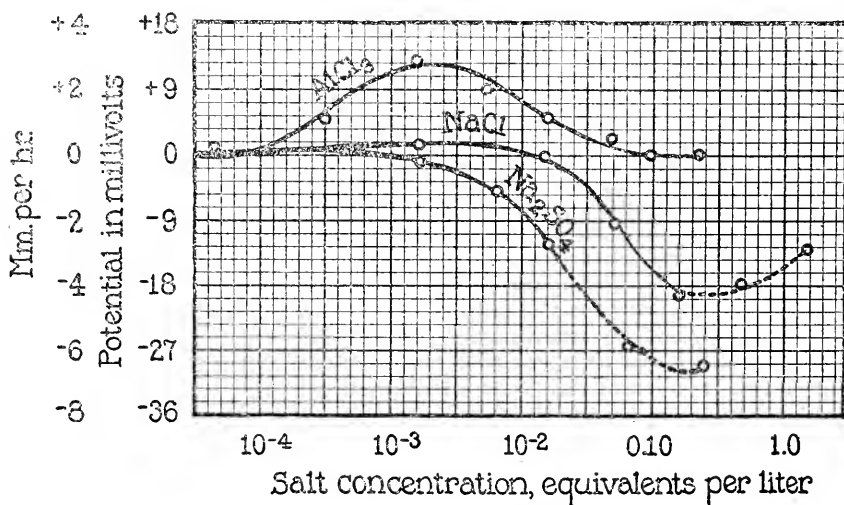


FIG. 7. Effect of salts on the potential and agglutination of Type D at pH 3 (0.001 N HCl).

is given, the pH was not regulated and the results are due in part to changes in the hydrogen ion concentration.

Inspection of the charts shows that in all experiments there is complete agglutination as soon as the potential is reduced below a value of about 15 millivolts (either positive or negative) provided the salt concentration is below 0.001 *N*. Below this salt concentration, therefore, the agglutination is seen to depend solely on the potential. Any substance which reduces the potential below about 15 millivolts will cause agglutination. There is another range of salt concentration above 0.10 *N* in which no agglutination occurs, although there is no measurable potential. Between these two ranges of salt con-

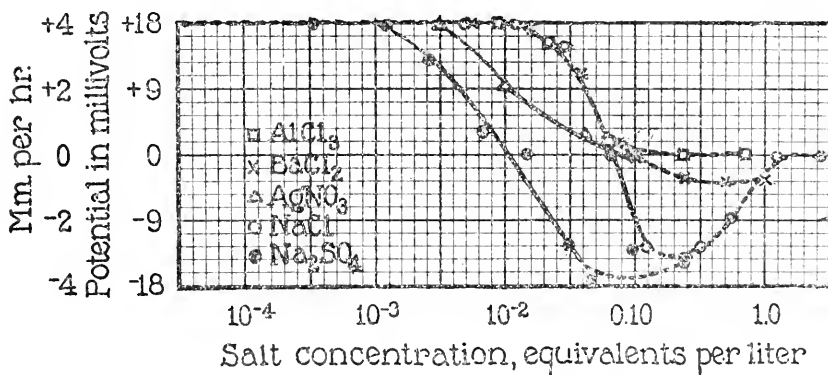


FIG. 8. Effect of salts on the potential and agglutination of Type D at pH 2.0.

centration there is a zone in which agglutination occurs at various potential levels. This is evidently the result that we would expect if the salt acted in low concentration primarily on the potential, and in high concentration on the cohesive force. There would be an intermediate zone in which the agglutination could not be predicted from either measurement alone. This explanation is borne out by the measurements of the cohesive force shown in Fig. 9. These show that the cohesive force is markedly decreased in concentrations of more than 0.01 *N*; *i.e.*, the range in which the critical potential begins to decrease. The figure shows that the effect on the cohesion is not connected with the valency nor with the electrical effects of the ions.  $\text{LaCl}_3$  is far more effective than  $\text{NaCl}$  in reducing the potential, but

less effective in reducing the cohesive force. The agglutination depends on both factors. It is possible, therefore, for all monovalent ions to affect the potential in the same way but to differ in their coagulating power. In order to predict the coagulating efficiency of a salt, it is therefore necessary to know the effect on both the potential and cohesion.

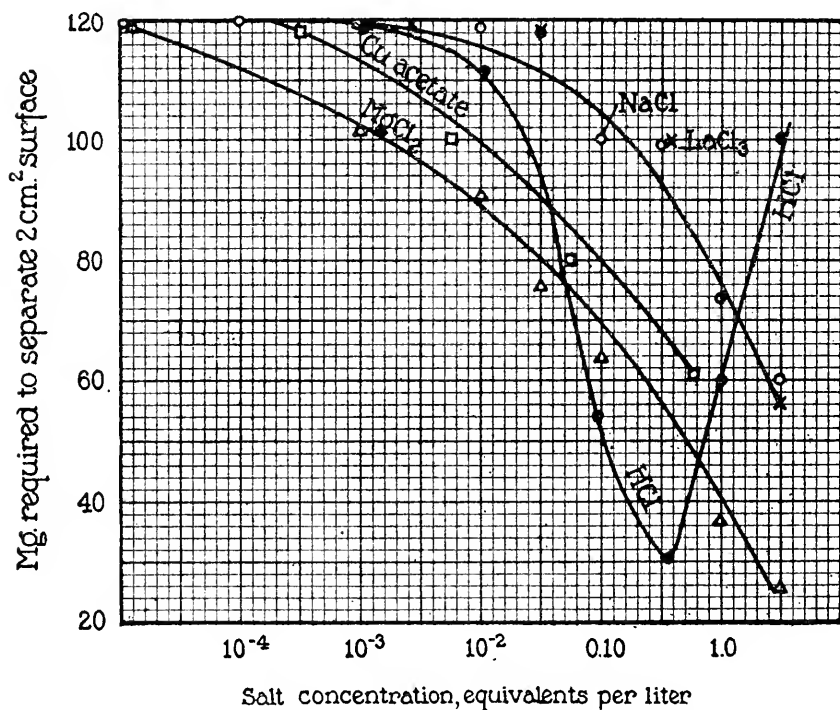


FIG. 9. Effect of salts and acids on the cohesive force between films of *B. typhosus*.

The HCl curve differs from the others in that the cohesion is *increased* in solutions of higher concentration than 0.3 N. This agrees with the agglutination test (Fig. 3) which shows a zone of agglutination at this concentration.

The experiments show the result usually obtained in such cases, that low concentrations of salt precipitate and higher concentrations stabilize again. They also show that this is due in most cases to the



fact that excess salt or acid confers a high potential upon the particles, of opposite sign to that in low concentration.

These various effects are all shown in the case of thorium chloride (Fig. 2). In concentration below  $5 \times 10^{-6}$  N no agglutination takes place since the potential is greater than 15 millivolts (the organisms being negative to the water) and the organisms are kept apart by the repulsion due to this potential. In concentrations between  $5 \times 10^{-6}$  and  $5 \times 10^{-5}$  N there is agglutination, since in this range the potential is less than 15 millivolts and the repulsion is therefore not sufficient to overcome the cohesion. In concentrations of from  $5 \times 10^{-5}$  to  $5 \times 10^{-1}$  the potential is greater than 15 millivolts (though of the opposite sign) and the suspension is again stable. At a concentration above 0.05 N the potential drops below 15 millivolts but agglutination does not occur since the cohesive force has also been reduced. A smaller potential is therefore sufficient to prevent agglutination. At a concentration of 0.10 N the potential is reduced practically to zero and agglutination again occurs. In still higher concentration the organisms are again stable due to a further decrease in the cohesive force.<sup>14</sup> The hydrochloric acid curve is interesting in that it shows a zone of agglutination in concentrated solutions ( $> 0.3$  N). This is due to the sudden increase in the cohesive force at this point as is shown in Fig. 9. This does not occur with the other chlorides and in the latter solutions no agglutination occurs in this range.

The stabilizing effect of sodium chloride in high concentration is shown more strikingly in Fig. 10,<sup>15</sup> which gives the result of adding increasing salt on the acid agglutination zone. The addition of

<sup>14</sup> According to O. Porges (*Centr. Bak., 1te Abt., Orig.*, 1906, xl, 133) agglutination occurs again in very strong salt solutions such as half saturated  $(\text{NH}_4)_2\text{SO}_4$ . This is probably a salting out phenomenon, due to a decrease in the forces between the surface of the particle and the liquid. For a review of the effect of salts on agglutination see Buchanan, R. E., *J. Bact.*, 1919, iv, 82. The experiment itself shows that this is a different phenomenon since in saturated  $(\text{NH}_4)_2\text{SO}_4$  agglutination occurs immediately whereas the type of agglutination studied in this paper requires considerable time.

<sup>15</sup> It will be noted that in this experiment the isoelectric point was about pH 4.2 while in others with *B. typhosus* (Fig. 3) it is about 3.5. This difference was noted several times and depends probably on the age and condition of the suspension.

0.01 N salt decreases the potential and broadens the agglutination zone slightly. More concentrated salts, however, although it reduces the potential still more, *decreases* the agglutination, since the cohesive force is now being reduced. In concentrations of more than 1.0 N no agglutination occurs. The salt also shifts the zone of agglutination to the acid side. This result has been obtained by Michaelis and Rona<sup>16</sup> with proteins.

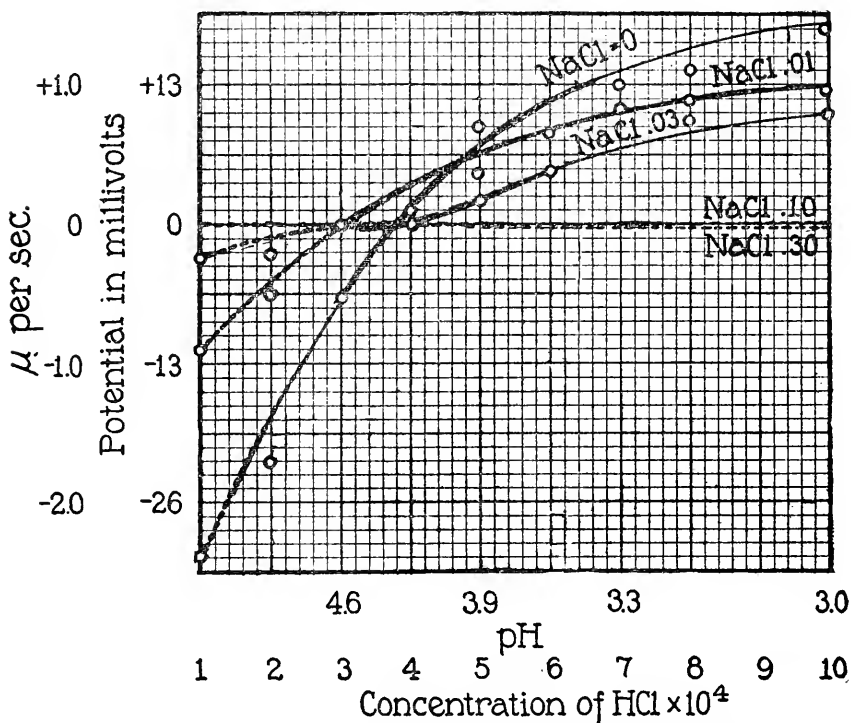


FIG. 10. Effect of NaCl concentration on the potential and agglutination of *B. typhosus* at the acid agglutination zone.

*Effect of the Salts on the Potential.*—The experiments show the familiar result that the effect is due to the oppositely charged ion and increases in general with the valence of the ion. The effect is not purely due to the valence since the hydrogen ion is far more active than the other monovalent ions. The result also depends on the nature of the

<sup>16</sup> Michaelis, L., and Rona, P., *Biochem. Z.*, 1919, xciv, 225.

suspension since the charge on the bacillus of rabbit septicemia may be reversed by sulfate or NaCl while with *Bacillus typhosus* suspension the charge is reduced but does not change in sign.

The experiments in Fig. 6 show clearly the reason for the characteristic difference in the stability of Types D and G of the rabbit septicemia bacillus.<sup>11</sup> Type D which is very stable has a high potential whereas the potential of Type G is very little more than the critical.

The same figure shows that the acid agglutination zone may be shifted markedly by the addition of other substances. Peptone for instance moves it far to the acid side (*cf.* Putter<sup>7</sup>) while immune body brings the isoelectric point to nearly 5. This point will be discussed more fully in the succeeding paper.

*Origin of the Potential.*—Loeb has shown,<sup>17</sup> in the case of a protein solution separated from a solution of electrolyte by a collodion membrane, that the charge on the protein solution can be quantitatively accounted for on the basis of Donnan's theory of membrane potentials. According to this theory, electrolytes affect the potential of a particle in two ways. (1) By combining chemically with the particle (for example hydrogen ions). The ion then becomes part of the molecule of which the particle (membrane) is composed. As a result the concentration of this ion differs on the opposite sides of the membrane and gives rise to a potential. This potential may be calculated by Nernst's formula from the concentration of the common ion on both sides of the membrane. The membrane behaves as a reversible electrode for this ion. (2) Ions which affect the distribution of the common ion without further chemical combination with the membrane. This mechanism will suffice to account for all the observations made in the course of this work, if it be supposed that other ions than the hydrogen ion may act by chemical combination.<sup>18</sup> The experiments are more complicated than those with a collodion membrane since the organisms are apparently more or less impermeable to ions.<sup>19</sup>

<sup>17</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 667; 1921-22, iv, 351; *Proteins and the theory of colloidal behavior*, New York and London, 1922, 120.

<sup>18</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 164, 165; *J. Gen. Physiol.*, 1921-22, iv, 463; also two papers in this number of the *Journal* which the writer has had the privilege of reading in manuscript form (*J. Gen. Physiol.*, 1921-22, iv, 741, 759).

<sup>19</sup> Shearer, C., *Proc. Cambridge Phil. Soc.*, 1916-19, xix, 263.

## SUMMARY.

1. Measurements have been made of the potential and of the cohesive force at the surface of *Bacillus typhosus* and the bacillus of rabbit septicemia in solutions of various salts and acids.

2. Electrolytes in low concentration (0.01 N) affect primarily the potential, and in high concentration decrease the cohesive force.

3. As long as the cohesive force is not affected, agglutination occurs whenever the potential is reduced below about 15 millivolts.

4. When the cohesive force is decreased the critical potential is also decreased, and in concentrated salt solution no agglutination occurs even though there is no measurable potential.

## THE STABILITY OF BACTERIAL SUSPENSIONS.

### III. AGGLUTINATION IN THE PRESENCE OF PROTEINS, NORMAL SERUM, AND IMMUNE SERUM.

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(Received for publication, May 24, 1922.)

It has frequently been noted that the addition of a small amount of certain substances, especially proteins, markedly affects the behavior of suspensions. It was found by Whitney and Blake<sup>1</sup> for instance that the sign of the charge of gold particles in the presence of gelatin could be reversed by acids, a result which did not occur without gelatin. The same effect has been noted by Loeb<sup>2</sup> in the case of colloidion membranes treated with different proteins. The membrane always acquires the isoelectric point of the protein used. It was found by one of the writers that peptone markedly affects the acid agglutination of the bacillus of rabbit septicemia. It has been shown in the preceding paper<sup>3</sup> that the isoelectric point was also displaced. This result had been noted by Putter.<sup>4</sup> The present paper contains the results of experiments on the effect of proteins and sera on the properties of suspensions of bacteria.

Fig. 1 shows the effect of various concentrations of egg albumin on the agglutination and charge of the bacillus of rabbit septicemia (Type D strain). The method of plotting is the same as in the preceding paper. Increasing the amount of egg albumin gradually shifts the curve to the alkaline side so that the isoelectric point is moved to pH 5.0 which is approximately that of egg albumin. In other

<sup>1</sup> Whitney, W. R., and Blake, J. C., *J. Am. Chem. Soc.*, 1904, xxvi, 1339.

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 659; 1921-22, iv, 213.

<sup>3</sup> Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 639.

<sup>4</sup> Putter, E., *Z. Immunitätsforsch., Orig.*, 1921, xxxii, 538. The same observation had been made independently by one of the writers De Kruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 345.

words, the particles act more and more like particles of egg albumin. The form of the curve is very similar to the curve found by Loeb<sup>5</sup> for the potential between a solution of egg albumin in a collodion sac and the surrounding solution. As was found in the experiments described in the preceding paper, agglutination occurs whenever the potential is reduced below a value of about 15 millivolts. The result of the addition of egg albumin is, therefore, that the agglutination zone is moved to the alkaline side and that at a pH of 3 the egg albumin stabilizes the suspension instead of precipitating it.

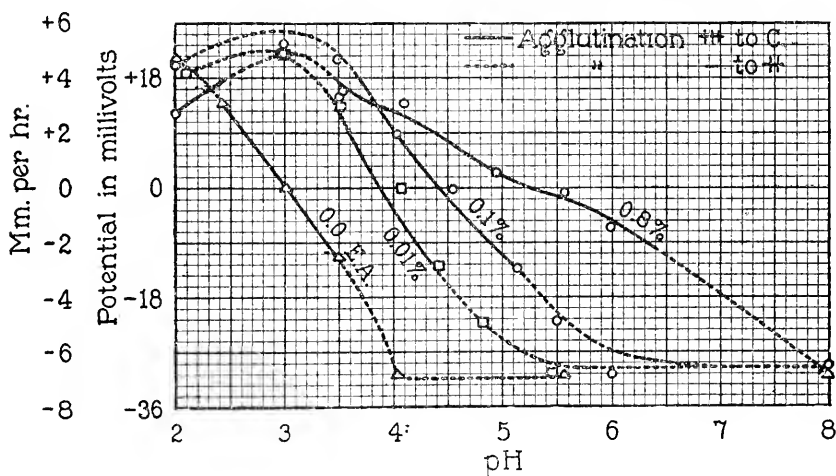


FIG. 1. Effect of the concentration of egg albumin at different pH on the potential and agglutination of Type D. pH adjusted with acetate buffers.

This is typical of the action of protective colloids and is due, as the figure shows, to the increase in the potential. The figure also shows that the amount of egg albumin required to agglutinate is a minimum near the isoelectric point of the suspension and increases as the pH is moved to the alkaline side. Similar experiments have been published by Eggerth and Bellows.<sup>6</sup>

Fig. 2 shows the effect of the addition of globin to a suspension of Type D; the isoelectric point is now shifted to pH 6.5 which is near

<sup>5</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922.

<sup>6</sup> Eggerth, A. H., and Bellows, M., *J. Gen. Physiol.*, 1921-22, iv, 669.

the isoelectric point of globin. Agglutination again occurs whenever the potential is less than 15 millivolts.

The effect of normal and immune serum on the pH curves of *Bacillus typhosus* is shown in Fig. 3.<sup>7</sup> The result is very similar to egg albumin. It will be noted that there is no marked difference between the immune and the normal serum and also that the isoelectric point is shifted to a pH of 4.7 in both cases. This was an unexpected result, since the isoelectric point of blood globulin is given by Mi-

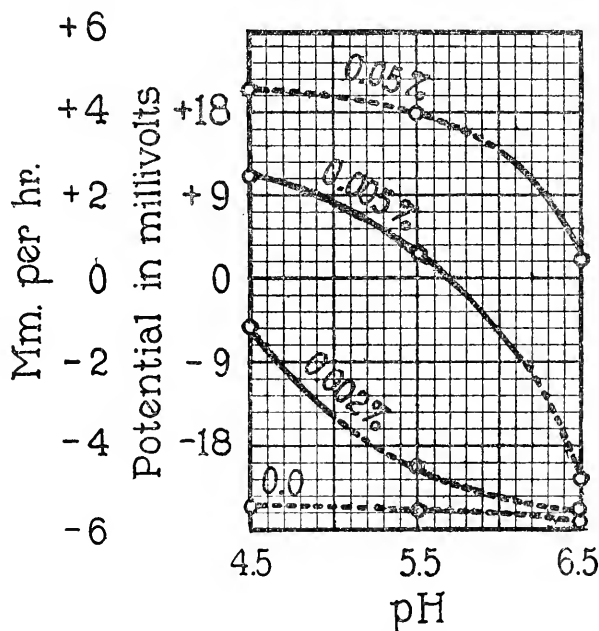


FIG. 2. Effect of the concentration of globin on the potential and agglutination of Type D at different pH. Acetate buffers.

chaelis<sup>8</sup> as 5.4 and the antibodies are known to be associated with the globulins. According to the present experiment, however, the substance in the serum which has the greatest effect on the charge of the

<sup>7</sup> Similar experiments have been made by Kōsaka and M. Seki, Communication to the Okayama Medical Society, *Okayama-Igakkwai Zasshi*, 1922, No. 386.

<sup>8</sup> Michaelis, L., *Die Wasserstoffionenkonzentration*, Berlin, 1914. It is doubtful if this can be considered the isoelectric point of pure immune body since traces of foreign proteins have such a marked effect.

organisms has an isoelectric point at about pH 4.7. The small difference in the concentration of normal and immune serum required to change the isoelectric point renders it improbable that this effect can be ascribed to the immune body.

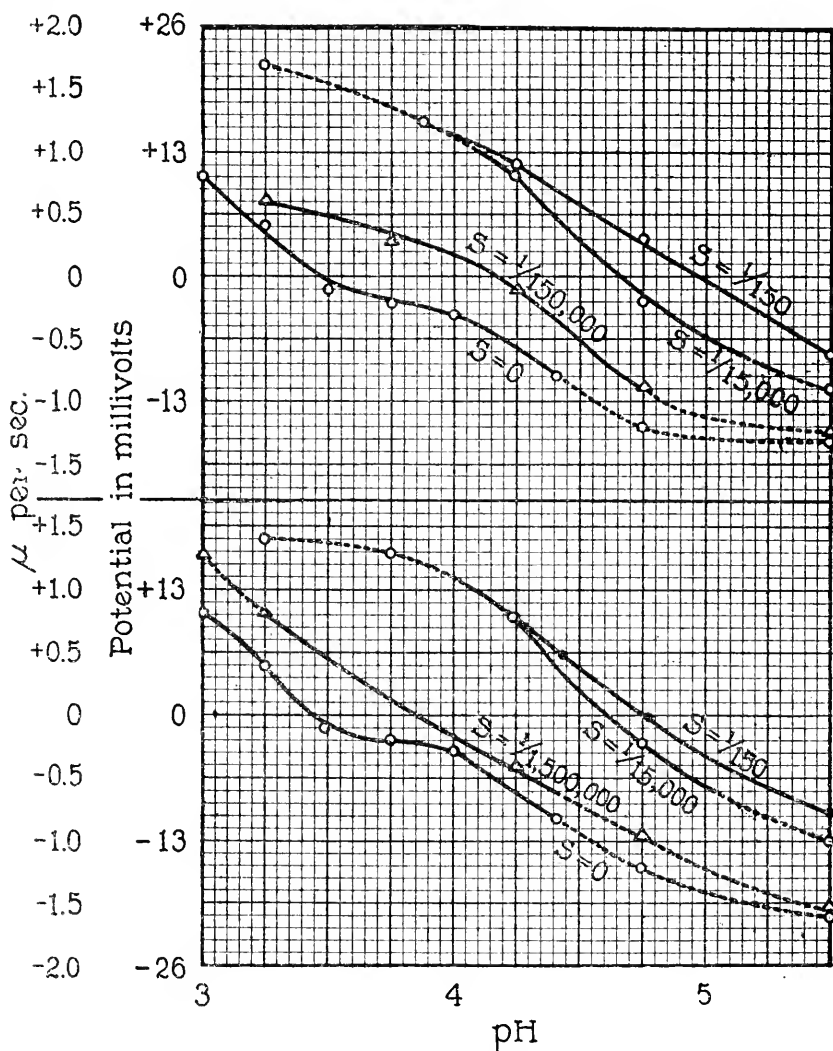


FIG. 3. Effect of different concentrations of normal (upper half) and immune serum (lower half) on the potential and agglutination of *B. typhosus* at different pH 0.01 N acetate buffer.



The stabilizing effect of the serum at pH 3 is likewise due to some constituent of the serum other than the immune body as is shown by the following experiment. A suspension of *Bacillus typhosus* was treated with an excess of immune serum in 0.10 N salt and then washed once with distilled water. The suspension was then added to

TABLE I.

*Agglutination of B. typhosus by Antityphoid Horse Serum at Various CH.  
G P. A. Buffer.*

pH	Concentration of serum.								Control. No serum.
	$5 \times 10^{-4}$	$2.5 \times 10^{-4}$	$1.25 \times 10^{-4}$	$6.2 \times 10^{-5}$	$3.1 \times 10^{-5}$	$1.55 \times 10^{-5}$	$7.8 \times 10^{-6}$	$3.9 \times 10^{-6}$	
8.5	C.	C.	C.	C.	C.	++	—	—	—
7.5	C.	C.	C.	C.	C.	++	+	—	—
6.0	C.	C.	C.	C.	C.	++	+	—	—
5.5	C.	C.	C.	C.	C.	++	+	—	—
5.2	C.	C.	C.	C.	C.	C.	+	Tr.	—
5.0	C.	C.	C.	C.	C.	C.	++	+	—
4.6	C.	C.	C.	C.	C.	C.	C.	C.	Tr. +
3.9	C.	C.	C.	C.	C.	C.	C.	C.	C.
3.3	+	+	+	++	++	C.	C.	C.	C.
2.7	+	+	+	+	+	+	+	+	+

*Normal Horse Serum.*

	pH	$2 \times 10^{-3}$	$1 \times 10^{-3}$	$5 \times 10^{-4}$	$2.5 \times 10^{-4}$	$1.25 \times 10^{-4}$	$0.6 \times 10^{-4}$
Normal horse serum 1.0 cc. + typhoid suspensions 1.0 cc	5.0	Tr.	Tr.	—	—	—	—
	4.7	+	Tr.	Tr.	—	—	—
	4.4	+	+	Tr.	+	++	++
	3.9	—	Tr.	+	C.	C.	C.
	3.3	—	—	+	+	++	++

pH 3 buffer. The result was instantaneous and intense agglutination, whereas Fig. 3 shows that in the presence of excess serum no agglutination occurs at a pH of 3.

Tables I to IV show the effect of the pH on the amount of normal serum and immune serum to cause agglutination of various organisms.

TABLE II.

*Agglutination of Types I and II Pneumococci by Pneumococcus Type I Antiserum at Various C<sub>H</sub>. G. P. A. Buffer.*

	pH	Dilution of serum.									Control. No serum.
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	
Pneumococcus Type I serum dilutions 1.0 cc. + pneumococcus Type I suspen- sions 1.0 cc.	8.5	C.	C.	C.	++	Tr.	-	-	-	-	-
	7.1	C.	C.	C.	++	+	-	-	-	-	-
	6.0	C.	C.	C.	++	+	-	-	-	-	-
	5.5	C.	C.	C.	++	+	-	-	-	-	-
	5.2	C.	C.	C.	C.	+	+	+	Tr.	Tr.	-
	5.0	C.	C.	C.	C.	++	+	+	+	Tr.	-
	4.6	++	++	C.	C.	C.	C.	++	+	Tr.	Tr.
	3.9	+	+	+	+	+	++	C.	C.	C.	C.
Pneumococcus Type I serum dilutions 1.0 cc. + pneumococcus Type II suspen- sions 1.0 cc.	8.5	+	+	-	-	-	-	-	-	-	-
	7.1	+	+	Tr.	-	-	-	-	-	-	-
	6.0	++	++	+	Tr.	-	-	-	-	-	-
	5.5	++	++	+	Tr.	-	-	-	-	-	-
	5.2	++	++	+	Tr.	Tr.	-	-	-	-	-
	5.0	++	++	+	+	Tr.	Tr.	-	-	-	-
	4.6	C.	C.	C.	++	+	+	Tr.	Tr.	Tr.	-
	3.9	+	+	C.	C.	C.	C.	C.	++	+	+

TABLE III.

*Agglutination of Type G Rabbit Septicemia Bacillus by Rabbit >G at Various C<sub>H</sub>.*

pH	Dilution of serum.											Control. No serum.
	1:10*	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	
7.9	++	++	+	+	-	-	-	-	-	-	-	-
7.5	++	C.†	++	+	Tr.	-	-	-	-	-	-	-
7.0	++	C.	C.	++	++	+	Tr.	-	-	-	-	-
6.5	C.	C.	C.	C.	++	+	+	Tr.	Tr.	Tr.	Tr.	Tr.
6.0	C.	C.	C.	C.	C.	++	+	+	Tr.	Tr.	Tr.	Tr.
5.5	C.	C.	C.	C.	C.	C.	++	++	+	+	+	+
5.0	C.	C.	C.	C.	C.	C.	C.	++	++	++	C.	C.
4.5	+	+	+	C.	C.	C.	C.	C.	C.	C.	C.	C.
2.2	-	-	-	-	-	-	-	-	-	-	-	-

\* Represent serum dilutions. Divide by 2 to obtain final dilution.

† C = Complete agglutination.

++ = Strong agglutination with sediment but turbid supernatant.

+ = Slight agglutination.

Tr. = Trace.

TABLE IV.

pH	Dilution of normal serum.										Control. No serum.
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
7.0	+	+	—	—	—	—	—	—	—	—	—
6.0	+	+	+	+	+	+	Tr.	Tr.	Tr.	Tr.	Tr.
5.5	+	+	+	+	++	++	++	++	++	++	++
5.0	+	++	++	++	++	++	C.	C.	C.	C.	C.
4.5	++	++	++	++	++	C.	C.	C.	C.	C.	C.
4.0	+	+	++	++	++	C.	C.	C.	C.	C.	C.
3.0	+	+	+	+	+	++	++	++	++	++	C.
2.0	—	—	—	—	—	—	Tr.	Tr.	Tr.	Tr.	Tr.

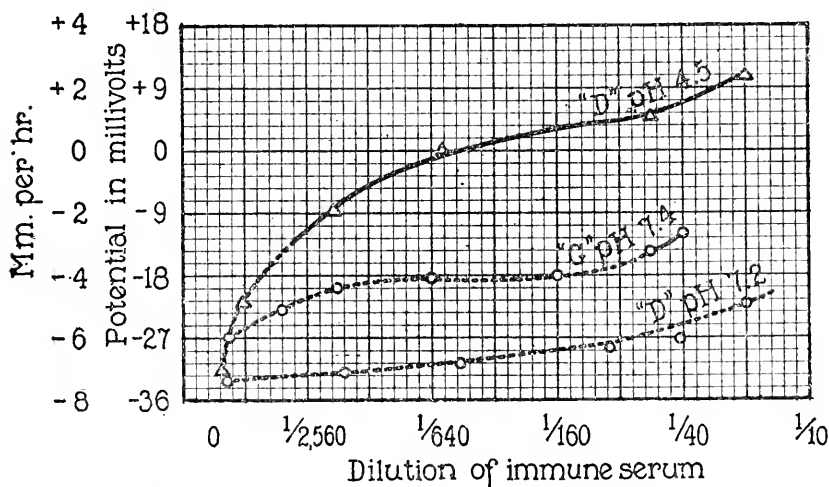


FIG. 4. Effect of immune serum on the potential and agglutination of Type D and Type G at different pH. G. P. A. Buffer.

The results are the same as with egg albumin. In every case the acid agglutination zone is shifted to the alkaline side, and the amount of serum required to agglutinate is a minimum near the isoelectric point of the organism. This result had been noted by Krumwiede and Pratt,<sup>9</sup> and by Michaelis and Davidsohn.<sup>10</sup> It is noticeable also that

<sup>9</sup> Krumwiede, C. K., Jr., and Pratt, J., *Z. Immunitätsforsch., Orig.*, 1913, xvi, 517.

<sup>10</sup> Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.

the difference between the normal and the immune serum becomes less and less marked as the pH approaches that of the acid agglutination zone of the organism.

Fig. 4 shows the effect of immune serum on the charge and agglutination of Types D and G. As in all the experiments, the agglutination becomes complete as soon as the charge is reduced below 15 millivolts. The figure shows that Type D is difficult to agglutinate because it has a fairly high charge at a pH of 7.2 and the effect of the immune serum is insufficient to reduce this to the critical value. Type G, however, has a lower charge and is much more readily agglutinated. Type D at a pH of 4.5 is easily agglutinated since at this pH the serum has a much greater effect on the charge.

#### *The Effect of Salts.*

Bordet<sup>11</sup> showed that salt greatly increased agglutination with immune serum. Porges,<sup>12</sup> however, found that with very powerful immune serum agglutination occurred even though the serum was dialyzed and no salt was present.

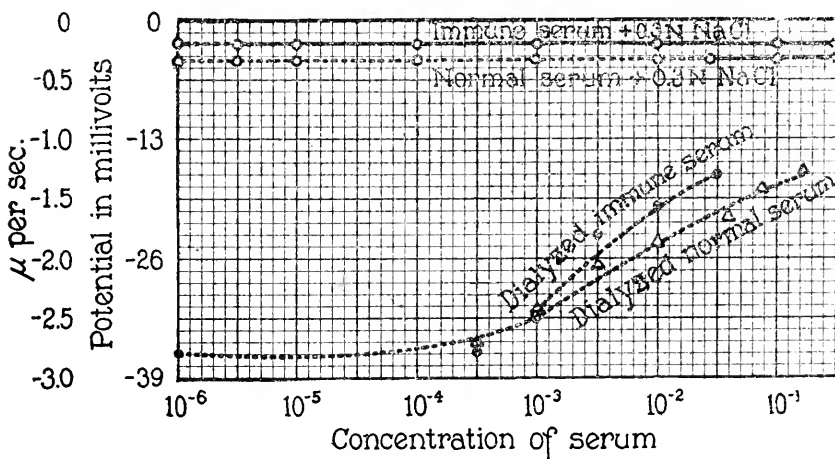


FIG. 5. Effect of dialyzed normal and immune serum on the potential and agglutination of *B. typhosus*. Upper curves show the effect in the presence of 0.3 N NaCl. The potential in these two curves is identical but they have been separated slightly in the figure in order to show the difference in the agglutination.

<sup>11</sup> Bordet, J., *Traité de l'immunité dans les maladies infectieuses*, Paris, 1920.

<sup>12</sup> Porges, O., *Centr. Bakt., 1 te Abt., Orig.*, 1905, xl, 133.

The effect of dialyzed normal and of powerful antityphoid horse serum on the potential and agglutination of *Bacillus typhosus* is given in Fig. 5. The two upper curves are the results in 0.3 N NaCl. There was no complete agglutination in the absence of salt and no marked difference between the normal and immune serum, although both affect the potential. (The serum was prepared by dialysis against distilled water and then dissolved by the addition of a small amount of NaOH. Conductivity measurements showed that the total concentration of salt was less than 0.001 N; *i.e.*, too small to cause the noted effect on the potential.) In the presence of salt, on the other hand, there is no effect on the potential but agglutination occurs in very high dilution with the immune serum and to a much less extent with the normal serum. This experiment shows that the effect of the serum on a suspension of bacteria in *concentrated salt* solution is not primarily on the charge but on the cohesive force. The serum raises the cohesive force and hence the critical charge to a value greater than the potential carried by the organism and they therefore agglutinate. The effect of the serum on the cohesion is shown in Fig. 6. The upper part of the figure shows that the addition of serum raises the cohesion to the value in distilled water; *i.e.*, it prevents the salt from decreasing the attractive force and thereby lowering the critical potential. The lower part of the figure shows the converse experiment; *i.e.*, the effect of salt on a film of washed, and of sensitized organisms. The salt decreases the cohesion of the washed organisms very markedly but has no effect on the cohesion of the film sensitized with serum.

The effect of varying both the salt and the serum concentration on the agglutination is shown in Table V. As the serum concentration is increased, the salt concentration in which complete agglutination occurs widens on both sides from 0.10 N. The lower limiting concentration of salt remains at about 0.01 N, however, and does not continue decreasing as the serum increases. In other words, the effect is not additive, but there is a critical concentration of serum beyond which there is little or no effect on the concentration of salt needed to agglutinate. This "critical" salt concentration corresponds to the point at which the charge on the organisms is about 10 millivolts; *i.e.*, just under the critical potential. This is the result expected if the

agglutinin forms a film on the surface of the organism. As soon as the layer is complete the addition of excess serum will have no effect. If this assumption is correct, it follows from Table V that agglutina-

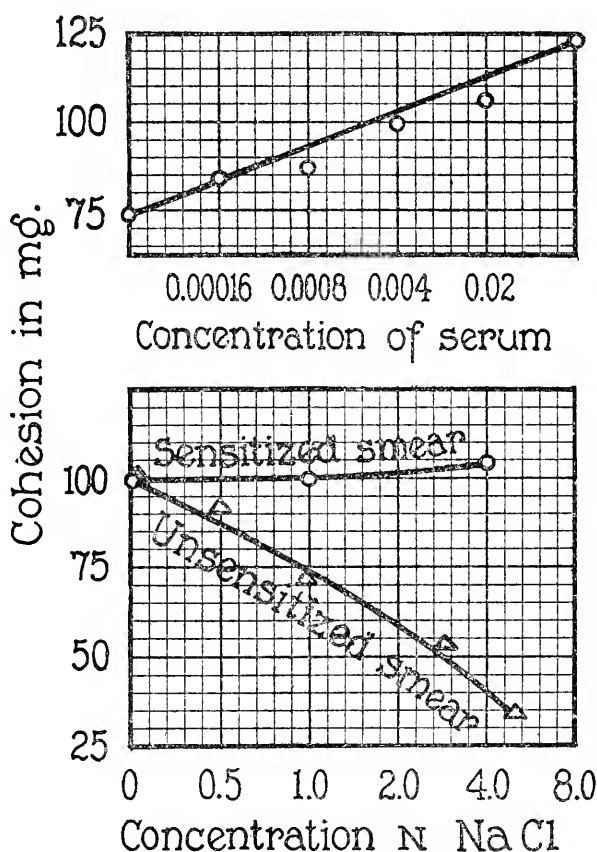


FIG. 6. Upper half, effect of the concentration of immune serum on the cohesive force between two films of *B. typhosus* in 0.10 N NaCl. Lower half, effect of concentration of NaCl on the cohesive force between, a, smears treated with immune serum and, b, untreated smears.

tion occurs when the surface is about one-eighth covered. The inhibiting effect of strong salt solution is, as usual, due to the decrease in the cohesive force, when the organisms are not completely covered with immune body.

It is evident from the foregoing that the agglutination may be considered as caused by the salt, as Bordet stated. The serum, however, does not *sensitize* the bacteria but *protects* it from the salt so that the latter does not reduce the cohesive force. If we study the effect of salts and acids on the agglutination and charge of organisms sensitized with immune serum, we should expect then to obtain curves similar

TABLE V.

*Influence of NaCl Concentration on Agglutination with Dialyzed Normal and Immune Serum.*

Concentration of immune serum	Agglutination after 24 hrs. at 20°C.								
	Concentration of NaCl.								
	0	0.001	0.003	0.01	0.03	0.1	0.3	1.0	1.4
1:150	+	+	+++	C.	C.	C.	C.	C.	C.
1:300	—	—	+++	C.	C.	C.	C.	C.	C.
1:600	—	—	+++	C.	C.	C.	C.	C.	C.
1:1,200	—	—	++	C.	C.	C.	C.	C.	C.
1:2,400	—	—	+	C.	C.	C.	C.	C.	++
1:4,800	—	—	—	++	C.	C.	C.	C.	+
1:9,600	—	—	—	+	++	C.	C.	++	—
1:19,200	—	—	—	—	+	C.	++	+	—
0	—	—	—	—	—	+	+	—	—
Concentration of normal serum.									
1:12	+	++	+++	C.	C.	C.	C.	C.	C.
1:24	—	—	+	C.	C.	C.	C.	+++	+++
1:48	—	—	—	+++	+++	+++	++	+	+
1:96	—	—	—	++	+++	++	+	+	—
1:192	—	—	—	—	+	++	+	—	—
$\mu$ per sec.	—2.5	—1.3	—1.0	—0.7	—0.4	—0.15	0	0	0
Millivolt potential.	—32	—17	—13	—9.0	—5.6	—0.19	0	0	0

to those given in the preceding paper with the exception that the stable zone in high concentrations of salt would not appear and the agglutination should be found to depend entirely on the potential. A summary of a number of such experiments is given in Fig. 7. The serum concentration was 1:500 in all cases; *i.e.*, in excess. The figure shows that with the exception of strong acid solution, complete agglutination occurred whenever the potential was reduced below 15

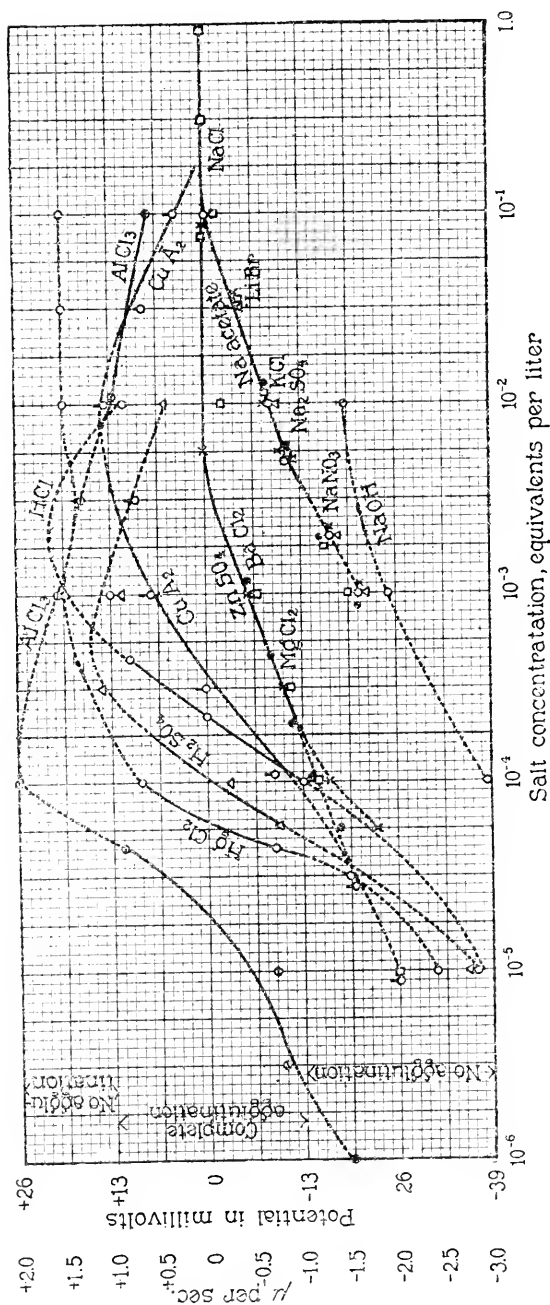


FIG. 7. Effect of the salt concentration on the agglutination and potential of *B. typhosus*, sensitized with immune serum.



millivolts, and there is no stable zone in concentrated salt. The effect of the strong acid is due partly to destruction of the antibody and partly to the fact that the combination of the antibody with the bacteria is less complete in acid solutions.<sup>13</sup>

The results also show that the effect of all monovalent cations (except hydrogen) was identical both as regards potential and agglutination. The valency and nature of the anion have no effect. This is the usual result when the particles are negative. The bivalent cations agglutinate in much lower concentration. The trivalent curves are not comparable owing to changes in the pH.

#### SUMMARY.

1. The addition of proteins or serum to suspensions of bacteria, (*Bacillus typhosus* or rabbit septicemia) at different pH widens the acid agglutination zone and shifts the isoelectric point to that of the added substance.

2. The amount of serum required to agglutinate is much less near the acid agglutination point of the organisms.

3. The addition of immune serum prevents the salt from decreasing the cohesive force between the organisms, and agglutination therefore is determined solely by the potential, provided excess immune body is present. Whenever the potential is decreased below 15 millivolts the suspension agglutinates.

<sup>13</sup> This point is taken up in the following paper.



## THE FLOCCULATION OF BACTERIA BY PROTEINS.

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The effect of the addition of proteins on the stability of suspensoid colloids has been the subject of numerous investigations. Ordinarily the proteins act to increase the stability of the suspension, hence their use as protective colloids. Under certain circumstances, however, they have been found to produce flocculation instead of protection. Neisser and Friedemann (1904) found that NaCl in a dilution incapable of producing flocculation by itself can coagulate mastic sol if one part of gelatin per million is present; blood serum, leech extract, and bacterial extract behaved in the same manner. Walpole (1913) reported that gelatin, albumin, and globulin, when added in high dilutions to oil emulsions or gold or mastic sols increased their sensitiveness to flocculation both by acids and salts. Brossa and Freundlich (1914) showed that dialyzed serum albumin, when added to  $\text{Fe}(\text{OH})_3$  sol, diminished the positive charge on the colloidal  $\text{Fe}(\text{OH})_3$  and caused it to be flocculated by concentrations of salt that were without effect upon the pure sol.

In the present investigation, the effect of pure proteins on the stability of bacterial suspensions at different H ion concentrations was studied. Several species of bacteria were used, but the most instructive results were obtained with a strain of *Bacterium coli*. This is because the point of optimum flocculation (the isoelectric point) for this organism lies in a very acid range, which leaves a wide interval between it and the isoelectric points of the different proteins studied. According to Beniasch (1912), *Bacterium coli* is not agglutinable by H ions. We have not found any recently isolated strains that would agglutinate in any of the buffer mixtures used by Beniasch; but most, though not all, strains of this organism will agglutinate in dilute acids in the absence of salt, at reactions ranging from pH 1.6 to 3.0. Putter

(1921) has also found *Bacterium coli* to agglutinate with acids in the absence of salt. The strain with which most of our work was done underwent a curious mutation about 2 months after it was first isolated. Our records show that on December 11 no agglutination took place in any of the acetate or lactate buffer mixtures; 3 days later the organism, with all its other characteristics unchanged, was found to agglutinate at pH 3.2 (negative at 3.0 and 3.5), both in the acetate and lactate buffer mixtures, but not in the phthalate buffers of Clark and Lubs (1917). No further change has been observed to the present time.

In all of these experiments, *Bacterium coli* was grown on beef extract peptone agar plates. The growth was suspended in 0.85 per cent NaCl, filtered through paper, centrifugated, then centrifugated three times out of distilled water. In most of the experiments, a temperature of 40°C. was employed. Flocculation was observed macroscopically; no test was considered positive unless definite macroscopic flocks were formed, which settled out to leave a clear supernatant fluid.

*Lactic Acid-Sodium Lactate Buffer Mixture.*

0.1 N lactic acid, cc.	0.6	1.2	2.4	5.0	10.0				
N lactic acid, cc.						2.0	4.0	8.0	16.0
Water, cc.	14.4	13.8	12.6	10.0	5.0	13.0	11.0	7.0	0
0.1 N sodium lactate, cc.	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
pH	4.7	4.4	4.1	3.8	3.5	3.3	3.0	2.7	2.4

*Acetic Acid-Sodium Acetate Buffer Mixtures.*

0.1 N acetic acid, cc.	0.6	1.2	2.4	5.0	10.0				
N acetic acid, cc.						2.0	4.0	8.0	16.0
Water, cc.	14.4	13.8	12.6	10.0	5.0	13.0	11.0	7.0	0
0.1 N sodium acetate, cc.	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
pH	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2

*Phosphate Buffer Mixtures.*

0.1 N potassium dihydrogen phosphate, cc.	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
0.1 N sodium hydroxide, cc.	0.37	0.57	0.86	1.26	1.78	2.36	2.96	3.50	
Water, cc.	14.63	14.43	14.14	13.74	13.22	12.64	12.04	11.50	
pH	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2	

Lactic acid-lactate and acetic acid-acetate buffer mixtures were made up according to the method of Beniasch (1912), which gives mixtures of uniform salt concentration (0.025 M). This uniformity of salt concentration is essential, as the salt effect is very high in some cases. The phosphate buffer mixtures were made up according to the directions of Clark and Lubs (1917), except that they are made twice as dilute, to make the salt concentration equal to that of the Beniasch buffer mixtures.

*Flocculation of Bacteria with Gelatin.*

Isoelectric gelatin was prepared by the method of Loeb (1919). Suspensions of *Bacterium coli* were incubated with varying dilutions of gelatin, both in buffer mixtures (Table I) and in the absence of salt (Table II).

TABLE I.

*Bacterium coli Suspension with Gelatin.*

1.0 cc. buffer mixture + 0.5 cc. gelatin solution + 0.5 cc. *coli* suspension.

Concentration of gelatin.	Acetate buffers.								Lactate buffers.		
0	—	—	—	—	—	—	—	+	—	—	—
1:4,000,000	—	—	—	—	—	—	—	+	—	—	—
1:400,000	—	—	—	—	—	—	+	+	—	—	—
1:40,000	—	—	—	—	×	×	×	×	+	—	—
1:4,000	—	+	×	×	×	+	—	—	—	—	—
1:400	—	—	+	—	—	—	—	—	—	—	—
pH	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2	3.0	2.7	2.4

Temperature = 40°C. × = agglutination within 30 minutes. + = agglutination within 6 hours.

In Table I it is to be noticed that with the highest concentration of gelatin, agglutination occurs only at pH 4.7, the isoelectric point of the gelatin. In the 1:4,000 dilution, the zone of flocculation widens, especially on the acid side, but it also includes the pH 5.0 tube. Beginning with the 1:40,000 dilution of gelatin, the zone of flocculation shifts over to the acid side, even becoming more acid than the flocculation zone of the control. It will be noted that there is an absence of flocculation with the higher concentrations of gelatin in the more acid tubes, forming a so called "proagglutinoid zone."

TABLE II.

*Bacterium coli* Suspension with Gelatin.Acid and water to make 1.0 cc. + 0.5 cc. gelatin solution + 0.5 cc. *coli* suspension.

Concentration of gelatin.	0.002 N HCl.							0.02 N HCl.			0.2 N HCl.		
	0	0.05 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.5 cc.	1.0 cc.	0.2 cc.	0.5 cc.	1.0 cc.	0.2 cc.	0.5 cc.	1.0 cc.
0	—	—	—	—	—	—	×(3.0)	×	×	×	×	×	×
1:4,000,000	—	—	—	—	—	—	×	×	×	×	×	×	×
1:400,000	—	—	—	—	—	×	×	×	×	×	×	×	×
1:40,000	—	—	—	—	—	×	×	×	×	×	×	×	×
1:4,000	—	—	—(5.2)	×	×	×	×	×	×	×	×	×	×
1:400	—	—	×	×	×	×	×	×	×	×	×	×	×

Temperature = 40°C. Flocculation complete in 30 minutes. Reactions were determined colorimetrically as soon as flocculation was evident; the figures in parentheses indicate the pH.

Cataphoresis experiments with a micro apparatus similar to that of Putter (1921) show that the bacteria in this zone carry a positive charge.

In Table II, the experiment is repeated in the absence of salt. The general results are similar to those found in Table I, except that the zone of flocculation of untreated *Bacterium coli* is found to lie between pH 1.6 and 3.0. It will be noted that when isoelectric gelatin is added to a suspension of *Bacterium coli*, the mixture is more alkaline than pH 4.7; this is because the bacterial suspension, though carefully washed and in pure water, maintains a pH of 6.8 to 7.0 even though unprotected from the CO<sub>2</sub> of the air.

The use of indicators in determining the H ion concentration of unbuffered or poorly buffered solutions calls for a word of explanation. Tizard (1910) has shown with methyl orange and methyl red that serious errors can be made. In a series of unpublished experiments made in collaboration with C. B. Coulter, in which all results were checked electrometrically, it was found that a carefully purified methyl red indicator could be used without serious error in ranges from pH 4.6 to 5.4. With the various sulfonephthalein indicators, which can be made up in aqueous solution, it was found that if the dye be adjusted so that its pH is not far from the pH of the solution tested, quite reliable results could be obtained. Thus, brom thymol blue indicator, adjusted so that when viewed in thin layers it matched the pH 6.8 standard, can be used over the range pH 6.4 to 7.2, without an error greater than 0.2 pH. Without adjustment of the indicator for particular ranges, the error may be much greater than this.

The results obtained in these experiments are strikingly similar to those found by Michaelis and Davidsohn (1912) in the precipitation of nucleic acid (isoelectric at about pH 0.7) by serum albumin, which is isoelectric at pH 4.8. Maintaining a constant concentration of nucleic acid, these authors found a concentration of albumin that produced a precipitate whose optimum occurred at pH 4.1 to 4.4. On lowering the concentration of albumin, the optimum now shifted to the acid side, and the optimal zone was greatly broadened. This is essentially like what we have described in Tables I and II. Michaelis and Davidsohn explained their results on purely electrical grounds. Nucleic acid is negatively charged at all reactions alkaline to pH

0.7; albumin is positively charged at all reactions acid to pH 4.8. At all intermediate reactions, the two colloids are oppositely charged; they tend to attract each other and neutralize their charges; when this occurs, their combination is isoelectric and flocks out. Moreover, at the acid end of this intermediate zone, the nucleic acid particle is weakly, whereas the albumin is strongly charged, hence a smaller amount of albumin will be needed to combine with and discharge the nucleic acid than at the other end of the zone, where the strongly charged nucleic acid would require larger amounts of the more weakly charged albumin to neutralize it. This view is capable of application to such phenomena as the flocculation of *Bacterium coli* by gelatin at pH 5.0 and 3.0 (Table I), points that lie outside of the intermediate zone. As these authors point out in another paper (1913), any solution of ampholyte at every pH exists in three forms,—as anion, as cation, and as undissociated molecule; it is the relative concentration of these three species that changes with H ion concentration. Cationic gelatin exists, therefore, at pH 5.0; anionic gelatin exists at pH 3.0. At pH 5.0, one might conceive that the bacterial cell (which is here negatively charged) would unite with the small amount of gelatin cations present; the gelatin cations being thus removed from solution, more would be liberated, this process continuing until a state of equilibrium between gelatin cations in solution and gelatin cations combined was reached. If the charge on the cell-gelatin complex is below a certain threshold value when equilibrium is reached, agglutination may occur, otherwise not. As it is the pH that determines the amount of gelatin cations in solution, it likewise determines the amount that can be combined when equilibrium is reached; hence it can be understood why agglutination, though occurring at pH 5.0, might not occur at pH 5.3. The same explanation would apply to the flocculation of *Bacterium coli* by proteins at reactions more acid than the flocculation zone of the untreated bacteria.

It is more difficult to apply this theory to the pro-zone observed in these experiments. Thus (Table I) when the gelatin concentration is 1:400, no agglutination of the bacteria occurs at reactions from pH 4.4 to 3.0, though more dilute gelatin causes flocculation. The bacteria in this zone are charged positively. If a primary electrical neutralization occurred, it must have been followed by further com-



bination with gelatin reactions, and this must have required a different mechanism. We cannot exclude the possibility that the primary cause of union between bacteria and protein is non-electrical (perhaps a surface tension phenomenon), and that electrical neutralization is a secondary effect which may or may not occur.

*Flocculation with Crystallized Egg Albumin.*

Crystallized egg albumin was obtained by the method of Hopkins and Pinkus (1898); this was dialyzed to remove the ammonium sulfate. Experiments were conducted in buffer mixtures only (Table III).

TABLE III.

*Bacterium coli* Suspension with Egg Albumin.

1.0 cc. buffer mixture + 0.5 cc. albumin solution + 0.5 cc. *coli* suspension.

Concentration of albumin.	Acetate buffer.	Lactate buffers.									
0	—	—	—	—	—	—	—	+	—	—	—
1:400,000	—	—	—	—	—	—	—	+	—	—	—
1:40,000	—	—	—	—	+	+	+	+	—	—	—
1:4,000	—	—	×	×	×	×	×	×	+	—	—
1:400	—	—	×	×	—	—	—	—	—	—	—
pH	5.0	4.7	4.4	4.1	3.8	3.5	3.3	3.0	2.7	2.4	

Temperature = 40°C. × = agglutination within 1 hour. + = agglutination within 4 hours.

Agglutination of this strain of *Bacterium coli* did not occur at pH 4.8, the isoelectric point of the albumin. A second strain of this organism was agglutinated at pH 4.7 by an albumin concentration of 1:150.

*Flocculation with Protalbumose and Heteroalbumose.*

These albumoses were prepared from Witte's peptone by the method of Pick (1898). The heteroalbumoses gave a clear solution when boiled and adjusted to pH 7.0; the protalbumose required no adjustment to give a clear solution.

The protalbumose was completely soluble at all reactions tested. The heteroalbumose, in a concentration of 1:400, was turbid at reac-

tions between pH 4.4 and 6.4, with a maximum turbidity at pH 5.3 and 5.6.

TABLE IV.

*Bacterium coli* Suspension with Protalbumose.

1.0 cc. buffer mixture + 0.5 cc. protalbumose solution + 0.5 cc. *coli* suspension.

Concentration of protalbumose.	Lactate buffers.									
0	—	—	—	—	—	+	—	—	—	—
1:4,000,000	—	—	—	—	×	×	—	—	—	—
1:400,000	—	—	—	—	×	×	×	×	—	—
1:40,000	—	—	—	—	×	×	×	×	—	—
1:4,000	—	—	—	×	×	×	×	—	—	—
1:400	—	×	×	×	×	×	×	—	—	—
pH	4.7	4.4	4.1	3.8	3.5	3.3	3.0	2.7	2.4	

Temperature = 40°C. All agglutinations were complete in 1 hour.

TABLE V.

*Bacterium coli* Suspension with Heteroalbumose.

1.0 cc. buffer mixture + 0.5 cc. heteroalbumose solution + 0.5 cc. *coli* suspension.

Concentration of heteroalbumose.	Phosphate buffers.			Acetate buffers.										Lactate buffers.		
0	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
1:4,000,000	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
1:400,000	—	—	—	—	—	—	—	—	—	—	+	+	+	+	—	—
1:40,000	—	—	—	—	—	—	—	+	×	×	×	×	×	×	—	—
1:4,000	—	—	—	—	—	×	×	×	×	×	×	×	×	—	—	—
1:400	—	+	×	×	×	×	×	×	×	×	+	—	—	—	—	—
pH	6.2	6.0	5.8	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2	3.0	2.7	2.4	

Temperature = 40°C. × = agglutination within 30 minutes. + = agglutination within 3 hours.

#### *Flocculation with Edestin.*

Crystalline edestin was prepared from hemp seed by the method of Osborne (1901). This substance was found to be practically insoluble in water or in the buffer mixtures used at reactions between pH 5.6 to 9.6. Rona and Michaelis (1910) report pH 6.9 as the

isoelectric point of edestin, though in a later paper Michaelis and Mendelssohn (1914) give it a value of pH 5.6.

In the experiment shown in Table VI, a 0.25 per cent suspension of edestin in distilled water was dissolved by the addition of minimal alkali; the solution was immediately distributed in the buffer mixtures and incubated at 40°C. for 30 minutes. The 1:1,600 dilution of edestin gave a small precipitate in the pH 5.3 tube, and a heavier flocculent precipitate in the tubes alkaline to this. *Bacterium coli* suspension was then added without stirring up the precipitate that had formed.

TABLE VI.

*Bacterium coli* Suspension with Edestin.

1.0 cc. buffer mixture + 0.5 cc. edestin solution + 0.5 cc. *coli* suspension.

Concentration of edestin.	Phosphate buffers.		Acetate buffers.								
0	—	—	—	—	—	—	—	—	—	—	+
1:1,600,000	—	—	—	—	—	—	—	—	—	—	+
1:160,000	—	—	—	—	—	—	—	—	+	×	×
1:16,000	—	—	—	×	×	×	×	×	×	+	—
1:1,600	—	+	×	—	—	—	—	—	—	—	—
pH	6.0	5.8	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2

Temperature = 40°C. × = agglutination within 30 minutes. + = agglutination within 6 hours.

*Flocculation with Hemoglobin.*

Crystallized oxyhemoglobin was prepared from the blood of the horse, the dog, and the guinea pig, some by the method of Hoppe-Seyler (1903), and some by the method of Dudley and Evans (1921). No marked differences in behavior were observed between these crystallized oxyhemoglobins and other solutions prepared by laking washed erythrocytes and centrifugating out the stroma. In the results obtained, there were some differences between the hemoglobins of different species of animals, and between the oxy- and methemoglobins of the same species.

It will be observed that in the most concentrated hemoglobin that we used (Table VII) the zone of flocculation extended only as far toward the alkaline side as pH 6.2 (in some experiments,

to pH 6.4). As we have observed that when we added a 1 per cent pure oxyhemoglobin solution to two or three volumes of *Bacterium coli* suspension, flocculation occurred without any adjustment of reaction, we suspected that salts interfered with the agglutination near the isoelectric point of the hemoglobin. In one experiment, where H ion concentrations were determined electrometrically, flocculation of the bacteria occurred in the absence of salt at pH 6.71 and points acid to this, the concentration of hemoglobin being 1:400.

It is also to be noted in Table VII that there is no pro-zone with the higher concentrations of hemoglobin, such as we have uniformly

TABLE VII.

*Bacterium coli* Suspension with Guinea Pig Oxyhemoglobin.

1.0 cc. buffer mixture + 0.5 cc. oxyhemoglobin solution + 0.5 cc. *coli* suspension.

Concentration of hemoglobin.	Phosphate buffers.				Acetate buffers.				
0	—	—	—	—	—	—	—	—	—
1:250,000	—	—	—	—	—	—	—	—	—
1:50,000	—	—	—	—	—	—	×	×	×
1:10,000	—	—	—	—	×	×	×	×	×
1:20,000	—	+	×	×	×	×	×	×	×
1:400	—	+	×	×	×	×	×	×	×
pH	6.4	6.2	6.0	5.8	5.6	5.3	5.0	4.7	4.4

Temperature = 20°C. × = agglutination within 1 hour. + = agglutination within 12 hours.

found with the other proteins studied. We did not extend the series more acid than pH 4.4, because the greenish brown color of the solutions showed that we no longer had oxyhemoglobin. When *Diplococcus pneumoniae* suspensions were used instead of *Bacterium coli*, a pro-zone was obtained; when the hemoglobin concentration was 1:400, the bacteria were charged positively at pH 5.0 and reactions acid to this, and remained non-agglutinated. When, instead of bacteria, we used an aqueous suspension of cellulose nitrate as substrate, hemoglobin in dilutions of 1:2,000 to 1:10,000 caused agglutination of the cellulose nitrate at reactions between pH 6.2 to 7.0 (in the absence of salt); with higher dilutions of hemoglobin, flocculation took place at more acid reactions.

The experiments described above establish the influence of proteins upon the stability of bacterial suspensions at different H ion concentrations, and relate this influence to the isoelectric point of the added protein and its concentration. It must be noted that bacteria are not the only suspensions that are effected by proteins in this way. We have tested the effect of proteins on sols of cellulose nitrate, cellulose acetate, and paraffin emulsions, with results that closely parallel our experiments with bacteria. Although Walpole (1913) does not relate his results to the isoelectric point of the protein, it seems clear, from the curve given by him, that gelatin, albumin, and globulin, affect the stability of oil emulsions and mastic and gold sols at different concentrations of H ions in the same manner that these substances affect bacterial suspensions. The same may be said of the action of albumin on  $\text{Fe}(\text{OH})_3$  sol at different salt concentrations, as reported by Brossa and Freundlich (1914). Of the same import are the observations of Putter (1921) that in the presence of peptone, acids change the sign of the charge on *Bacterium coli*, and of Coulter (1922) who shows that erythrocytes in contact with specific sensitizer, or even with normal homologous or heterologous sera, agglutinate most promptly when the pH is such that the euglobulins of these sera are isoelectric.

Loeb (1920) has shown that when collodion membranes are treated with proteins, a combination takes place between the collodion and the protein, and the membrane becomes isoelectric near the H ion concentration at which the protein is isoelectric. Reactions acid to this point now charge the membrane positively. It is obvious that the effect of proteins upon bacteria (and probably upon other suspensions and emulsions) is of the same nature. Where concentrations of about 0.25 per cent of protein are used, the bacteria agglutinate at or near the isoelectric point of the protein; increasing the H ion concentration beyond this point causes the original charge upon the bacteria to be reversed, and prevents agglutination.

#### SUMMARY.

1. The effect of adding pure proteins to bacterial suspensions at different H ion concentrations has been studied.

2. The zone of flocculation of protein-treated bacteria bears a significant relationship to the isoelectric point of the protein used. With the higher concentration of protein, agglutination occurs at or near the isoelectric point of that protein; at reactions acid to this, the bacteria carry a positive charge and are not agglutinated. With diminishing concentration of protein, the zone of flocculation shifts toward and goes beyond that characteristic of the untreated bacteria. This occurs both in the presence and absence of salts.

3. A diversity of other suspensions, such as sols of gold, mastic, cellulose nitrate, cellulose acetate,  $\text{Fe}(\text{OH})_3$ , oil emulsions, and erythrocytes, have been found by ourselves and others to exhibit a similar altered stability when treated with proteins in the same way.

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## FURTHER STUDIES ON EOSIN HEMOLYSIS.

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It is a well known fact that if a dilute solution of a photosensitive substance such as eosin is added to a suspension of washed red blood cells and the mixture is exposed to sunlight, hemolysis of the red cells promptly takes place, while no action is observed when the mixture is kept in the dark. Busck (1) and later Sellards (2) found that the addition of certain substances such as blood serum and egg white to solutions of photobiologic sensitizers results in decreasing or completely inhibiting the toxic action, but no protection is afforded to cells by the addition of glucose, starch, or gelatin. Recognizing that there are fundamental differences in the chemical make-up of those substances which afford protection and those which do not, Schmidt and Norman (3) carried out experiments to determine the relation between the amino-acid content of the protein molecule and protective action. They found that eosin hemolysis can be prevented by the addition of tyrosine, tryptophane, and proteins which contain these amino-acids. Certain other organic compounds which contain the hydroxyphenyl ring also afford protection. They pointed out that the inability of gelatin to protect red blood cells against eosin hemolysis is due to the absence of the above essential amino-acids. As a tentative explanation of this phenomenon, they consider that the protection afforded by certain substances against the photodynamic effect of eosin may possibly be due to the absorption of the active rays by the protective substance.

Since the publication of these experiments, we noted a striking similarity between the substances which protect red blood cells against hemolysis by eosin, and the substances which were found by Gortner and Holm (4) to react with the Folin and Denis (5) phosphotungstic-phosphomolybdic reagent to give a characteristic blue color. These

substances were found by Gortner and Holm to include tyrosine, tryptophane, uric acid,  $\alpha$ -methyl indole, and ferrous iron. Abderhalden (6) states that the list also includes oxyproline and oxytryptophane. The property which is possessed in common by all of these substances is that they are easily oxidizable.

Experimental work was accordingly undertaken to determine whether inorganic reducing substances in addition to tyrosine, tryptophane, and proteins which contain these amino-acids in the molecule can afford protection to red cells against eosin hemolysis, and our results appear to answer this question in the affirmative. As in the previous work, 0.5 cc. of a 5 per cent saline suspension of red blood cells (ox or sheep) was placed in each of a number of small test-tubes, and to each, 1 cc. of a 1:10,000 dilution of eosin (Grübler's) in salt solution was added. The substances to be tested for protective action were likewise made up in normal saline solution in the concentrations, as given in Table I, and the reaction was adjusted to approximate neutrality. The tubes were placed in direct sunlight for 30 minutes and after exposure they were immediately placed in the ice chest. The tubes were inspected at the end of several hours to determine the amount of lysis which had taken place. Control tubes which were kept in the dark eliminated factors other than that of photodynamic action. The experimental results are given in Table I. They indicate that inorganic reducing substances afford marked protective action to red blood cells against eosin hemolysis. The list of inorganic reducing substances which may be used in experiments of this type is limited, since many of the best reducing agents such as ferrous chloride, ferrous sulfate, and ferrous ammonium sulfate, yield solutions of high acidity, and when these are added to red cells the latter are agglutinated. Oxyproline and oxytryptophane were not available for experimental work. It is doubtful whether the former substance can protect red cells against the toxic action of eosin, since gelatin, which contains 14 per cent (7) of this substance, lacks protective ability. Marked protection was shown by each of the two preparations of histidine. Both gave a trace of blue color when tested by the Folin and Denis reagent, indicating the possible presence of tyrosine. Valine, serine, proline, creatinine, and cinnamic acid afford no protection, while marked protection is afforded by skatole and tryptophane.



Since the action of the protective substance appears to be that of a reducing agent, it seemingly follows that eosin hemolysis is a phenomenon involving oxidation. Hemolysis may be wholly prevented by placing the eosin-red cell mixture in a highly evacuated glass tube or by saturating the cells with illuminating gas or hydrogen. These observations are in agreement with the statement of Sellards, that an atmosphere of hydrogen is as effective as total darkness in preventing

TABLE I.

*The Effect of the Addition of Certain Substances on the Hemolysis of Red Cells by Eosin.*

Substance added.	Concentration.*	Result.
Sodium chloride (control).....		Complete hemolysis.
Valine.....	M/10	" "
Serine.....	M/10	" "
Proline.....	M/10	" "
Cinnamic acid.....	M/10	" "
Creatinine.....	M/30	" "
Tryptophane.....	M/30	No hemolysis.
Skatole.....	Saturated solution.	" "
Sodium sulfite.....	M/30, M/90	" "
Sodium thiosulfate.....	M/30	" "
Ferrous lactate.....	M/30	" "
Potassium ferrocyanide.....	M/30	" " Some hemolysis over night.
Histidine 1.....	M/20	No hemolysis.
Histidine 2.....	M/20	" "

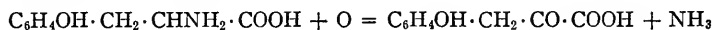
\* Sufficient NaCl was added to each of these solutions to make them isotonic.

the toxic action of eosin in sunlight. It must be admitted that in certain instances the reducing agent may react with the fluorescent substance and in this way partially inhibit its toxic action. Thus, when sodium sulfite is added to a solution of eosin and the mixture is exposed to sunlight, the latter substance is rapidly reduced to fluorescein.

The action of sunlight in accelerating oxidative reactions is a well known phenomenon. Bilirubin when exposed to sunlight is oxidized to biliverdin (8), many of the vegetable oils are oxidized by light, and the bleaching of the triphenylmethane dyes (9) is a phenomenon in

which the dye itself appears to catalyze the oxidation which is accelerated by the sun's rays. We have noted that the bleaching of eosin solutions when exposed to sunlight may be markedly inhibited by the addition of tryptophane, while alanine, glycocoll, and phenylalanine afford little or no protection.

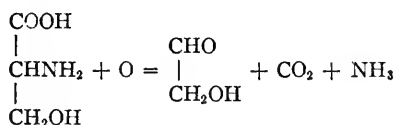
Three possible reactions may conceivably take place in the oxidation of the protective protein or amino-acid. The first is oxidative deamination which yields an  $\alpha$ -ketonic acid. For tyrosine the reaction is represented by the equation:



This is a universal reaction and represents a step in the normal catabolism of the amino-acids. The protective action against eosin hemolysis afforded by tyrosine and tryptophane cannot be due to this reaction, since it is not at all specific for these two amino-acids. Moreover, ammonia, which itself possesses hemolytic properties, is set free in the reaction. It is possible that the latter substance may be a factor which is concerned in eosin hemolysis. Experimental work, however, does not appear to support this hypothesis. A series of test-tubes containing 100 mg. each of glycocoll, alanine, and tryptophane, dissolved in 5 cc. of normal salt solution and 1 cc. of 1:10,000 eosin was exposed to sunlight for 1 hour. At the end of this time the eosin was decolorized in the tubes containing glycocoll and alanine. The ammonia was taken up with permutit and subsequently set free by the addition of NaOH and the solutions were Nesslerized. A trace of ammonia was found in each instance while none was evident in the control tubes which had been kept in the dark. After 4 hours exposure, the tryptophane solution gave an ammonia content which corresponds to approximately 0.1 cc. of a 0.03 normal solution, about half of the amount necessary to hemolyze completely the dosage of red cells. A series of test-tubes, each containing 1 cc. of a 0.1 normal solution respectively of glycocoll, alanine, phenylalanine and tryptophane, and 1 cc. of 1:10,000 eosin solution was exposed to sunlight for 1 hour, and after exposure 0.5 cc. of a 5 per cent saline suspension of washed sheep cells was added to each tube, the mixtures were shaken and placed in the ice chest. Only a trace of hemolysis was shown by the tubes after standing over night. In this connection it might be

mentioned that the ammonia, which is formed as a result of the oxidation of the amino-acid, is probably not free but is combined with the ketonic acid.

Neuberg (10) studied the mechanism of the reaction which takes place when solutions of certain amino-acids to which uranium salts have been added are exposed to sunlight. In addition to oxidative deamination, he found that  $\text{CO}_2$  was split off from the carboxyl group yielding an aldehyde, the reaction being similar to that which takes place when an amino-acid is oxidized with  $\text{H}_2\text{O}_2$ . When serine is exposed to sunlight or is oxidized with  $\text{H}_2\text{O}_2$ , glycol aldehyde is formed according to the reaction:



This reaction like the one discussed previously is universal, and fails to explain the specific reducing action of tyrosine and tryptophane.

It is a well recognized fact that benzene and a large number of aromatic substances of varied types undergo substitution of the hydrogen atoms in the nucleus to a more or less marked extent, and *in vitro* this reaction can be brought about by ozone,  $\text{H}_2\text{O}_2$ , and by photochemical action (11). We have attempted to test the possible application of this reaction to the subject of eosin hemolysis. Suspensions of washed red cells were treated respectively with  $\text{H}_2\text{O}_2$  (saline solution of the neutralized product),  $\text{H}_2\text{O}_2$  plus a small amount of catalase, and  $\text{H}_2\text{O}_2$  plus platinum black. In each instance the oxyhemoglobin was converted into methemoglobin, but hemolysis did not take place. On saturating red cells with ozone a similar result was obtained. Tubes containing red cells to which platinum black and colloidal palladium were added, showed no hemolysis after exposure for a half hour to sunlight. Evidently hemolysis cannot be brought about with the aid of  $\text{H}_2\text{O}_2$ . The fact that substances which contain either the hydroxyphenyl ring which apparently facilitates the introduction into the nucleus of other OH groups, or the indole ring which is easily oxidized to indoxyl and then to indigo blue (Abderhalden (12) has noted that tryptophane and adrenalin are sensitive to light), can afford protection

to red blood cells against the toxic action of eosin, leads us to believe that we are dealing with a special type of oxidation which is markedly accelerated by fluorescent substances. The highly specific action of tyrosine and tryptophane as reducing agents likewise indicates that these amino-acids which are contained in the protein molecule are attacked and undergo oxidation as a result of the photodynamic action of eosin. It is not possible at this time to state how far this oxidation proceeds or the mechanism whereby lysis takes place. It appears logical to assume that the oxidation concerns itself with the proteins of the stroma and this results in the necrosis of the cell (13).

Bovie (14) has demonstrated that coagulation of proteins can be brought about by exposure to ultra-violet light. This reaction is presumably one of denaturation (15) and does not involve oxidation. To eliminate the possibility of denaturation being a factor in eosin hemolysis, the following experiment was carried out: a test-tube containing 2 cc. of horse serum and 0.1 cc. of 1:1,000 eosin was exposed to sunlight for a period of 4 hours and subsequently incubated at 37°C. There was no visible coagulation.

#### SUMMARY.

Additional experimental work on the subject of eosin hemolysis has been carried out. This indicates that red cells may be protected against the toxic action of eosin in sunlight by the presence of inorganic reducing agents. It is pointed out that a marked parallelism exists between the substances which react with the Folin and Denis reagent and the compounds which afford protection to red cells against the photodynamic action of eosin. The property which is possessed in common by all of the substances is that they are easily oxidized, and their ability to protect red cells lies in their power of reduction. The toxic action of eosin probably involves the oxidation of tyrosine and tryptophane which are contained in the protein molecules of the stroma.

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# THE CALIBRATION OF THE OSTERHOUT RESPIRATORY APPARATUS FOR ABSOLUTE QUANTITIES OF CARBON DIOXIDE.

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In the course of a piece of work on the amount of carbon dioxide produced by sea anemones it was found desirable to use the respiratory apparatus devised by Osterhout (1918), and as it was necessary in this work to determine the absolute quantity of carbon dioxide eliminated by these animals, an attempt was made to calibrate this apparatus for such determinations. The method finally adopted seems to be of enough general importance to justify a brief statement of it.

The Osterhout apparatus consists of a closed system in which the air is made to circulate by means of a pump. The air passes from a chamber, in which the organism is confined and in which consequently the carbon dioxide is produced, either directly to a glass tube containing an indicator in solution or indirectly to this tube through a U-tube filled with fragments of sodium hydroxide. In the direct course the air containing the carbon dioxide is forced to bubble through the indicator solution, thus causing it to change its tint. In the indirect course the air is relieved of its carbon dioxide by contact with the sodium hydroxide, and, after purification, it is bubbled through the indicator thus washing out the contained carbon dioxide and bringing the indicator back to its original color. From the tube containing the indicator the air returns by a direct course through the pump to the chamber in which the organism is confined. A figure of the apparatus is given by Osterhout (1918). In taking readings with this apparatus the time in seconds required for the indicator to change from one tint to another as compared with standard solutions of known pH value is recorded and the rate

of this change is assumed to be identical with that of the excretion of carbon dioxide by the organism.

In calibrating the apparatus various means were used to produce a constant and measurable flow of carbon dioxide as a substitute for that from the organism. About 100 cc. of a normal solution of sulfuric acid were poured into the chamber in which the organisms had been kept, and into this were dripped at a constant rate solutions of sodium bicarbonate of different but known concentrations. The rates of change in the indicator did not correspond to the calculated rates of liberation of carbon dioxide from the solutions, and it soon becomes apparent that all the carbon dioxide produced was not liberated but that part of it remained behind in the solution. The dripping of weak acid on dry sodium bicarbonate or on marble also failed to yield consistent results.

The method of calibration finally adopted was that of making mixtures of carbon dioxide and atmospheric air and of introducing them at a known rate into the apparatus in place of the organism. Into a narrow graduated glass receiver filled with water carbon dioxide was bubbled till a given volume at atmospheric pressure was attained, whereupon, the remainder of the receiver was quickly filled with atmospheric air and the whole closed. This operation was carried on over a water bath so quickly that the solution of the carbon dioxide in the water was reduced to a negligible amount. Mixtures of gases containing 0.4, 1, 2, and 4 per cent of carbon dioxide were used. Each of these mixtures was introduced through a connecting tube into the chamber of the apparatus in which the organism had been kept by running into the gas receiver at a known rate a minute stream of mercury and thus driving out the mixture of gas. As the glass gas receiver was graduated, the amount of gas driven out of it in any given time could be easily read off by observing the levels at which the mercury stood in it as shown by the scale on its wall. Thus a constant and measurable supply of carbon dioxide was substituted for that produced by the organism. In this form of procedure the rate of change in the indicator corresponded very closely to the rate of introduction of carbon dioxide. Since the volume of mixed gases introduced into the apparatus in any test was very small in comparison with the total volume of the apparatus,



no attention was given to the slight increase of density that must have ensued in the course of a test by introducing gas into an apparatus already filled with air at atmospheric pressure.

The indicator used in these tests was an aqueous solution of phenolsulfonephthalein (Hynson, Westcott, and Dunning), and the times in seconds necessary to change its tint from that characteristic for pH 7.78 to 7.36 (Osterhout and Haas, 1918), at the four concentrations of carbon dioxide used, are given in Table I.

The steps necessary to determine the amount of pure carbon dioxide in ten-thousandths of a milligram delivered per second to

TABLE I.

*Times in Seconds Needed to Change a Standard Solution of Indicator (Phenolsulfonephthalein) from pH 7.78 to 7.36 by the Introduction into the Osterhout Respiratory Apparatus of Four Mixtures of Gases Containing Respectively 0.4, 1, 2, and 4 Per Cent of Carbon Dioxide.*

Concentration of carbon dioxide, per cent.....	0.4	1	2	4
Time to change indicator from pH 7.78 to 7.36, sec.....	569 559 563 558 564	218 228 214 221 229	106 104 106 108 106	58 58 56 58 57
Average time.....	562.6	222.0	106.0	57.4
Rate $\left(\frac{1}{\text{average time}}\right)$ .....	0.00178-	0.00450+	0.00943+	0.0174+

the apparatus are shown in Table II. In the table are given: (A) the percentage concentration of the carbon dioxide mixture, followed by the time (B) required to deliver 10 cc. of this mixture to the apparatus. By dividing 10 cc. by the number of seconds needed to deliver that amount of gas to the apparatus, the volume of gas delivered per second was found (C). By multiplying this volume by the appropriate per cent indicating the proportion of the impure carbon dioxide contained in the given mixture, the several volumes of impure carbon dioxide delivered were determined (D). By absorbing with sodium hydroxide in a graduated tube a sample of

the carbon dioxide used, it was found that this gas was pure to the extent of 97.2 per cent and on introducing this correction into the calculation, the volume of pure carbon dioxide delivered per second

TABLE II.

*Observed Times in Seconds for the Delivery of 10 Cc. of the Four Gas Mixtures Containing Respectively 0.4, 1, 2, and 4 Per Cent of Impure Carbon Dioxide (B), Calculated Amounts in Hundred-Thousandths of a Milligram of Pure Carbon Dioxide Delivered per Second to the Apparatus (F), and Calculated Constants for the Apparatus (G).*

A. Impure carbon dioxide, per cent.....	0.4	1	2	4
B. Observed times for delivery of 10 cc. of mixed gas, sec.....	297	296	293	298
C. Calculated volume of gas delivered per sec. ( $= \frac{10}{297}$ etc.), cc.....	0.003367+	0.003378+	0.003413-	0.003356-
D. Calculated volume of impure carbon dioxide delivered per sec. ( $= C \times A$ ), cc.....	0.00001347-	0.00003378+	0.00006826-	0.00013424-
E. Calculated volume of pure carbon dioxide delivered per sec. ( $= D \times 0.972$ ), cc.....	0.00001309+	0.00003283+	0.00006635-	0.00013048+
F. Calculated weight (in hundred-thousandths of a milligram) of pure carbon dioxide delivered per sec. ( $= E \times 1.75984 \times 100,000$ ).....	2.304-	5.777+	11.676+	22.962+
G. Constants for apparatus (= Average time, Table I, $\times F$ ).....	1,296+	1,282+	1,238-	1,318+

in each of the four tests was calculated (E). To change the quantitative determinations of carbon dioxide from volumes to weights, the volumes of this gas in cubic centimeters were multiplied by 1.75984,

the weight in milligrams of 1 cc. of carbon dioxide at 24°C. and 762 mm. of barometric pressure, the conditions of the test. The result of this operation was then multiplied by 100,000 to permit the final number to be expressed in hundred-thousandths of a milligram (F). In this way the weight of carbon dioxide delivered per second and expressed in hundred-thousandths of a milligram was arrived at.

In the use of this apparatus it is assumed that equal concentrations of carbon dioxide produced the same color tints in the indicator. Since in all four tests the readings were always begun at the same tint and ended at another tint always the same, it follows that the amount of carbon dioxide delivered per second in each test multiplied by the number of seconds over which the test extended ought to yield a constant. And such seems to be the case, as is shown in the last line of Table II in which the product of the average times (Table I) by the weights of carbon dioxide delivered per second are given. These constants vary from 1,238—to 1,318+ and average 1,283.5; they are a measure of the amount of carbon dioxide necessary in this particular piece of apparatus to change the indicator from the tint characteristic of pH 7.78 to that for pH 7.36.

Another way of expressing these relations is shown in the graph (Fig. 1) in which the weights in hundred-thousandths of a milligram of carbon dioxide delivered are plotted as abscissæ, and the rates at which the indicator changed as ordinates. As the plotting shows, the relations are clearly linear.

In using these results to determine the absolute amount of carbon dioxide produced by an organism, it is convenient to express them in the form of an equation thus:

$$K = T \times W$$

where  $K$  is the constant already determined for the apparatus,  $T$  the time in seconds for the change in the indicator from one pH value to the other, and  $W$  the weight of carbon dioxide in hundred-thousandths of a milligram delivered per second. As the constant and the time in the operation described are the known factors and the weight the desired one, the most convenient statement of the equation is

$$\frac{K}{T} = W$$

and it is in this form that I have used it in calculating the weights of carbon dioxide produced by sea anemones. Thus in one instance a sea anemone weighing 0.5 gm. brought about the necessary color change in the indicator in 424.8 seconds. This animal must, therefore, have produced  $1,283.5/424.8$  or  $3.0+$  hundred-thousandths of a milligram of carbon dioxide per second. Another one also weighing 0.5 gm. brought about the same change in 420.4 seconds and by a similar calculation can be shown to have produced  $3.1 -$  hundred-

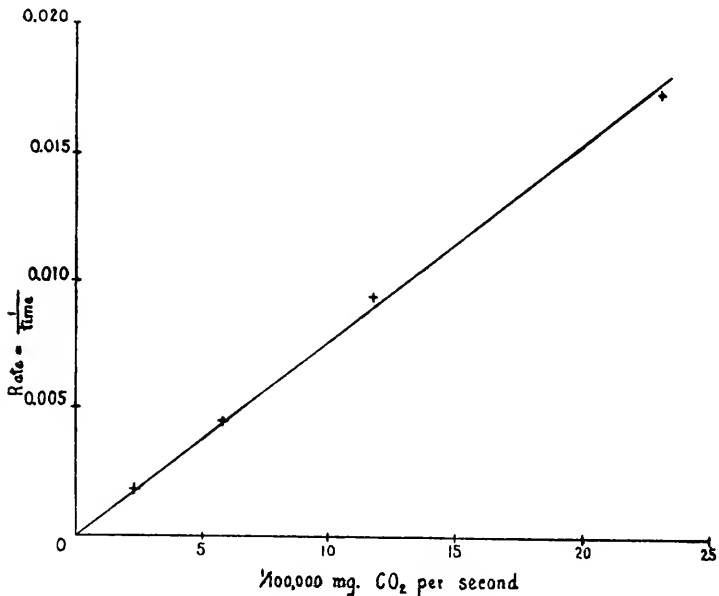


FIG. 1.

thousandths of a milligram of carbon dioxide per second. The two animals together changed the indicator over the requisite range in 213 seconds which when used as a basis of calculation yield  $6.0+$  hundred-thousandths of a milligram of carbon dioxide per second or almost exactly twice that of the sea anemones taken separately. Thus the proposed formula affords an easy means of calculating the absolute amount of carbon dioxide excreted when the time of the indicator change and the apparatus constant are known.

It must be clear from the foregoing account that each apparatus will have its own constant, and that anyone who wishes to calibrate such an apparatus for the absolute amount of carbon dioxide produced will first have to determine this constant by some such method as that already described. Once, however, having made this determination, the formula already given may be used with perfect accuracy for the calculation of the absolute amount of carbon dioxide produced. Since the constant is a measure of the amount of carbon dioxide necessarily present in a given piece of apparatus, in order that a standard change in the indicator shall take place, and since this amount is spread through the space contained within the apparatus, it is probable that the constant is directly related to the volume of the apparatus and will be small in an outfit of small volume and large in one of large volume, but such relations have not as yet been worked out.

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# STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS.

## I. THE SOLUBILITY OF CERTAIN PROTEINS AT THEIR ISOELECTRIC POINTS.

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In 1899 W. B. Hardy observed that "under the influence of a constant current the particles of proteid in a boiled solution of egg white move with the negative stream if the reaction of the fluid is alkaline; with the positive stream if the reaction is acid" (1). The protein in the "boiled solution of egg white" had, of course, been denatured, but Pauli (2, 3) later investigated the direction of migration of undenatured serum albumin in an electric field, and found that it, too, moved toward the cathode in an alkaline solution and toward the anode in an acid solution. Presumably the charge on the protein was negative in alkaline, but positive in acid solution.

Michaelis was the first investigator to determine the hydrogen ion concentration at which the migration of a protein changed its direction. He studied serum albumin, and found that the change was abrupt (4). It occurred within exceedingly narrow limits. At a hydrogen ion concentration of  $2.1 \times 10^{-5}$  serum albumin migrated toward the cathode. At smaller hydrogen ion concentrations than  $1.9 \times 10^{-5}$  it migrated toward the anode. At  $2.0 \times 10^{-5}$  the protein appeared to be in an isoelectric condition (5) and this hydrogen ion concentration was accordingly termed the isoelectric point.

The movement in an electric field of particles charged with respect to their surrounding medium is termed cataphoresis. From cataphoresis the sign, and the average magnitude, of the charge can be determined, but not its nature or origin. The nature of the charge of the protein molecule will be discussed in a later section of this paper from a theoretical point of view.

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The isoelectric points of many proteins have been inferred from the change in the direction of their migration in an electric field. The method of cataphoresis has, however, not been found universally applicable. Certain proteins, notably glutenin, casein, and the prolamines are only very slightly soluble at certain hydrogen ion concentrations. If acid, or, under other circumstances, alkali, be added to their solutions a precipitate appears, increases in amount, and finally disappears if the hydrogen ion concentration is sufficiently altered. Michaelis observed (6) that such proteins, like the denatured albumin that Hardy first studied (1), migrated to the cathode on the alkaline side of the precipitation zone, and to the anode on the acid side. Accordingly he concluded, in part from theoretical considerations to be discussed later, that the protein precipitated in the isoelectric condition, and that the maximum precipitation coincided with the isoelectric point. The phenomenon of precipitation has therefore generally been substituted for the phenomenon of cataphoresis in determining the isoelectric point of this class of substances; the isoelectric point being considered coincident with the point of maximum precipitation or flocculation.

Still a third class of proteins, the globulins, although like the last group they are also precipitated at certain hydrogen ion concentrations in the absence of any appreciable concentration of electrolytes, are readily soluble in solutions of neutral salts. In this class also the precipitation zone has been found to correspond to the isoelectric zone. That is to say, isoelectric globulin was found to migrate toward the cathode when dissolved by acid, toward the anode when dissolved by base. When serum globulin was dissolved in a salt solution, however, neither Michaelis (6) nor Chick (7), who confirmed his observations, was able to detect a charge on the protein molecules by cataphoresis.

Since globulins are dissolved by neutral salts over a considerable range of hydrogen ion concentrations—and the nature of this phenomenon and its relationship to the isoelectric point of the globulins we shall reserve for a subsequent communication—practise has consisted in reducing this range by the removal of salt, and then either in noting the point of maximum precipitation of the globulin, or the limiting hydrogen ion concentrations at which migration occurred in an electric field.



Experience has shown that neither of these methods of determining the isoelectric point of a globulin is altogether satisfactory. It is true that the precipitation of a globulin passes through a sharper maximum the freer it is from salt. Freedom from salt, at least as applied to the proteins is, however, relative. Probably no protein has even been prepared that was completely "ash-free." Moreover, three distinct sources of error may make, and in the experience of the writer have made the point of maximum precipitation of a protein appear at a hydrogen ion concentration other than its isoelectric point.

The first of these is the presence of another protein with a slightly different isoelectric point. The observed precipitation is in this case the sum of the precipitations of the two proteins. As a result the zone is usually widened, and the point of maximum precipitation shifted in the direction of the isoelectric point of the second protein. The magnitude of the shift, and therefore of the error, depends upon the difference in the isoelectric points of the two proteins, upon their relative concentrations, and upon their relative solubilities.

The presence of salts containing either bivalent or trivalent cations or anions may also lead to error. For multivalent ions, even in low concentration, shift the point of maximum precipitation to a hydrogen ion concentration other than the isoelectric point of the protein. Hardy (8), Mellanby (9), Osborne and Harris (10), and later Hopkins and Savory (11), have shown that the solvent action of salts upon globulins increases greatly with the valence of their ions. Moreover, cations of high valence are more effective in dissolving globulins on one side of the isoelectric point and anions on the other. For this reason the salts of monovalent acids and bivalent bases, (or the salts of monovalent bases and bivalent acids) exert a greater solvent action upon globulins at certain hydrogen ion concentrations than at others. This also results in a shift in the precipitation zone.

In greater concentration salts precipitate proteins of all classes. The precipitating action of electrolytes, like the solvent action, increases greatly with the valence (1). Michaelis has recently shown theoretically and experimentally how the presence of salts of high valence shifts the point of maximum precipitation of proteins (12).

Finally, I have observed that even uni-univalent salts of the type of NaCl in relatively low concentrations, may shift the precipitation

(though possibly not the flocculation) and therefore the apparent isoelectric point of tuberin and serum globulin to a slight degree. The reason for this appears from a theoretical consideration of the nature of the charge on the protein molecule.<sup>1</sup>

As a result of the unsatisfactory nature of the experimental methods that have been employed in determining the isoelectric point of the slightly soluble proteins, and in view of the manifest importance and significance of the isoelectric point for the interpretation of other aspects of the physical chemistry of the proteins, it seems necessary to base these studies upon better criteria of the identity of the proteins under investigation.

### *Theoretical.*

Hardy had been led to examine the cataphoresis of denatured albumin from a consideration of the contemporary conceptions of colloidal chemistry. Picton and Linder (15) had "established . . . . that the direction of the movement of colloidal particles under the influence of an electric current is determined by their chemical nature," and Hardy concluded<sup>2</sup> that "proteid molecules seem therefore to act as basic or acid particles according to the circumstances in which they find themselves."

In the same year in which Hardy published these conclusions Bredig (16) extended the theory of electrolytic dissociation to the case of molecules that act both as acids and as bases; that is, to amphoteric electrolytes. The amphoteric nature of the amino-acids and also of the proteins had already been recognized (17, 18, 19).

<sup>1</sup> If the assumption be made that the charge on the protein is due to its dissociation as an amphoteric electrolyte, then the cation of any salt will depress the dissociation of any protein through its common ion on the alkaline side of the isoelectric point, and the anion on the acid side. Sørensen (13) has used this phenomenon as a method of determining the isoelectric point of egg albumin; the isoelectric point coinciding with the hydrogen ion concentration that is unchanged by the addition of a neutral salt. I have attempted to apply the method to tuberin (14) but it is not entirely suitable to the globulins. Moreover, even where the method is applicable for the determination of the isoelectric point in this way, the effect of a common ion in depressing dissociation and thereby affecting solubility need not be identical on both sides of the isoelectric point.

<sup>2</sup> Hardy (1), p. 297.

Because of their simpler structure, the amino-acids have been found more suitable than the multivalent proteins for the experimental verification of dissociation theory. Accordingly the generalizations, in terms of which we have attempted to explain the dissociation of the proteins, were first worked out for their prototypes, the amino-acids.

Amino-acids possess at least one amino group, and at least one carboxyl group. The amino groups dissociate as bases and combine with acids, much as does ammonia. The carboxyl groups dissociate after the manner of organic acids and combine with bases. As a result of its amphoteric nature an amino-acid can form internal salts (20, 21, 22, 23). If the amino group of one acid combines with the carboxyl group of the next with the loss of a molecule of water, a dipeptide is formed which in turn is an amphoteric substance. Polypeptides, in which many amino-acids are combined in this manner, have been synthesized by Emil Fischer (24). They simulate proteins in behavior, and suggest that the free valences in the protein molecule are in all probability derived at least in part from the free groups of the amino-acids.

The strength of these groups in a number of amino-acids was first determined by Winkelblech (20) in 1901, from the hydrolysis of their acid and basic salts. In the hands of Walker (21), however, Winkelblech's data revealed the relations that obtain between the degree of dissociation of an amphoteric electrolyte and its strength as an acid and as a base. Following Walker in the main, therefore, and in part the more recent investigators<sup>3</sup> who have amplified his conceptions, we shall deduce the fundamental equations for the dissociation of an amphoteric substance, for our present purposes a protein, P.

Let  $\text{HPOH}$  represent the undissociated protein molecule. The protein can dissociate into an hydrogen ion and a protein anion,



and into an hydroxyl ion and a protein cation,




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<sup>3</sup> Notably Hardy (1), Sørensen (13), Lundén (23), Michaelis (25), Henderson (26), Robertson (27), Pauli (28), and Loeb (29).

if the protein be considered as a monovalent acid, giving rise to but one hydrogen ion, and a monovalent base, giving rise to but one hydroxyl ion. That the multivalent protein can under certain restricted circumstances be considered as uni-univalent we shall presently show.

The mass law equation defining the dissociation of the protein as an acid may be written

$$\frac{(H^+) (POH^-)}{(HPOH)} = K_a \quad (3)$$

and as a base

$$\frac{(HP^+) (OH^-)}{(HPOH)} = \frac{(HP^+) K_w}{(H^+) (HPOH)} = K_b \quad (4)^4$$

Equations (3) and (4) on multiplication yield an expression for the dissociation of the pure ampholyte, or rather for the square of the dissociation

$$\frac{(HP^+) (POH^-)}{(HPOH) (HPOH)} = \frac{K_a \cdot K_b}{K_w} \quad (5)$$

The method of deriving this equation involves the elimination of the hydrogen ion concentration from both equations (3) and (4). As a result the pure ampholyte can exist at only one hydrogen ion concentration; namely, that at which the ampholyte dissociates to form as many anions as cations. This point has been identified as the point at which the migration of protein in an electric field changes in direction; that is, as the so called isoelectric point. It has been defined by Michaelis as the point at which

$$(HP^+) = (POH^-) \quad (6)$$

and is determined by substituting in equation (6) the value of  $(POH^-)$  in equation (3) and of  $(HP^+)$  in equation (4). We then obtain

$$\frac{K_a (HPOH)}{K_b (HPOH)} = \frac{(H^+)^2}{K_w} \quad (7)$$

and if we assume that the undissociated protein molecule is the same on both sides of the isoelectric point (30) we obtain

$$\frac{K_a}{K_b} = \frac{(H^+)^2}{K_w} \quad (8)$$

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<sup>4</sup>  $(H^+) (OH^-) = K_w$

At the hydrogen ion concentration that corresponds to the isoelectric point a definite ratio obtains between the total amount of the acid dissociation and the total amount of the basic dissociation. This ratio obtains irrespective of how many acid valences of different strength or how many basic valences of different strength are involved. For at any one hydrogen ion concentration the number of active acid constants may be considered equal to one constant,  $K_a$ , and the active basic constants to another constant,  $K_b$  (31). For this reason the multivalent protein may be treated as a simple ampholyte at its isoelectric point. We shall so consider it in this paper, since we are, for the moment, only concerned with the solubility of certain proteins at their isoelectric points.

Michaelis pointed out that the precipitation of a protein was at a maximum at its isoelectric point, since dissociation was at a minimum (25). This follows from equation (5) if we define maximum precipitation as minimum solubility, and assume that protein is as a rule more soluble in the dissociated than in the undissociated state. According to this conception a protein should be more soluble the greater its amphoteric constants. I have shown (31) that this was the case for different classes of proteins in a previous communication, by using the acid- and the base-combining capacity of a protein as a measure of its amphoteric strength.<sup>5</sup>

In order to pursue this investigation further it was necessary to correlate the solubility of proteins with their dissociation. This involved accurately determining the solubility of a number of proteins at their isoelectric points, and quantitatively distinguishing between the concentrations of dissociated and undissociated protein. Let the solubility of a protein, P, be S. This solubility is made up of the concentration of the undissociated protein molecule and of the dissociated protein ions, in the saturated solution. We may write

$$S = (\text{HPOH}) + (\text{HP}^+) + (\text{POH}^-) \quad (9)$$

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<sup>5</sup> In practise this was estimated by titrating electrometrically 1 gm. of protein with NaOH and HCl and measuring the rate of change of the hydrogen ion concentration (by the slope of the tangent to the titration curve) at the isoelectric point. These estimates are therefore subject to revision when sufficiently accurate data of the molecular weights of the proteins are available.

provided the protein is quite uncombined with acid or with base. If a part of the protein is combined with a base B, however, or a part with an acid A, we must write<sup>6</sup>

$$S = (\text{HPOH}) + (\text{HP}^+) + (\text{POH}^-) + (\text{BP}) + (\text{PA}) \quad (10)$$

But let us first consider the case of an uncombined protein at its isoelectric point. Its degree of dissociation is given by equation (5) as equal to  $\sqrt{\frac{K_a \cdot K_b}{K_w}}$ . This ratio is, as we have shown above, a constant for any protein at its isoelectric point. Walker showed that the degree of dissociation of an amino-acid as acid or as base,  $\frac{(\text{HP}^+) (\text{POH}^-)}{(\text{HPOH})}$ , was a constant, independent of dilution from conductivity data.

In the case of a protein that is relatively insoluble at its isoelectric point the undissociated protein HPOH may be assumed to have a definite solubility. Moreover, this molecule dissociates, as we have seen, to form a protein cation  $\text{HP}^+$ , and a protein anion  $\text{POH}^-$ . The solubility determined experimentally must be the sum of the concentrations of the undissociated protein molecule and of the protein ions. But if the concentration of the undissociated molecule is constant, the degree of dissociation must also be constant at the isoelectric point. Solubility must therefore be constant so long as the solution is saturated with respect to undissociated protein.

Experiments now to be described have shown that this is the case when the protein is uncombined with base or acid. The solubility of casein and of the two globulins, tuberin and serum globulin, that have thus far been investigated, was found to be constant at the respective isoelectric points of the proteins, when the amount of protein precipitate with which the solution was in heterogeneous equilibrium was varied within wide limits.<sup>7</sup>

Only at the isoelectric point was solubility independent of the amount of the protein in the system. At greater hydrogen ion concen-

<sup>6</sup> I am indebted to Prof. S. P. L. Sørensen for first calling my attention to the convenience of treating solubility as an unknown function of concentration.

<sup>7</sup> If the amount of the protein precipitate was increased beyond a certain point, a secondary effect slightly increasing solubility was detected.

trations the protein is combined with acid, and at smaller with base.<sup>8</sup> In either case the solubility is no longer equal to the sum of the undissociated protein molecule and its resultant ions, but to the sum of these and the protein acid or basic compound. Equation (10) and not equation (9) then obtains. Moreover, the degree of dissociation of the compound of a protein with either a strong acid or a strong base is not the same as that in comparison with the dissociation of

#### CORRECTION.

On page 704, Vol. iv, No. 6, for  $\frac{(\text{HP}^+)(\text{POH}^-)}{(\text{HPOH})}$  read  $\frac{(\text{HP}^+)(\text{POH}^-)}{(\text{HPOH})^2}$ .

the isoelectric point of a protein. This method is free from the errors that often enter into the determination of the minimum in solubility of a protein, though theoretically, and in practise, the two methods are capable of giving the same result. But, since the presence of a foreign protein (unless it has the same solubility and the same isoelectric point), or of multivalent cations or anions, or of a neutral salt, may shift the point of minimum solubility of a protein in the manner and for the reasons that have already been described, the presence of these impurities renders impossible the determination of the true isoelectric point. Upon their removal, however, protein will dissolve in water to a constant and characteristic extent, and will dissociate to a constant and characteristic extent. The hydrogen ion concentration due to this dissociation is also a characteristic of each protein, and has come to be known, as we have seen, as its isoelectric point.<sup>9</sup>

<sup>8</sup> Experiments that will be reported in a subsequent communication suggest that a protein can exist in combination with acid and base even at its isoelectric point.

<sup>9</sup> In practise the hydrogen ion concentration due to the dissociation of pure protein is determined with great difficulty because of its very small solubility.

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These observations, therefore, present a new method of determining the isoelectric point of a protein. This method is free from the errors that often enter into the determination of the minimum in solubility of a protein, though theoretically, and in practise, the two methods are capable of giving the same result. But, since the presence of a foreign protein (unless it has the same solubility and the same isoelectric point), or of multivalent cations or anions, or of a neutral salt, may shift the point of minimum solubility of a protein in the manner and for the reasons that have already been described, the presence of these impurities renders impossible the determination of the true isoelectric point. Upon their removal, however, protein will dissolve in water to a constant and characteristic extent, and will dissociate to a constant and characteristic extent. The hydrogen ion concentration due to this dissociation is also a characteristic of each protein, and has come to be known, as we have seen, as its isoelectric point.<sup>9</sup>

<sup>8</sup> Experiments that will be reported in a subsequent communication suggest that a protein can exist in combination with acid and base even at its isoelectric point.

<sup>9</sup> In practise the hydrogen ion concentration due to the dissociation of pure protein is determined with great difficulty because of its very small solubility.

For all these reasons we have come to consider the solubility of a protein at its isoelectric point as a fundamental physicochemical constant, characterizing and identifying the protein under investigation. We will attempt in subsequent communications to relate this constant to the amphoteric dissociation of the proteins.

#### EXPERIMENTAL.

##### *The Purification of Protein.*

The three proteins whose isoelectric solubilities have thus far been determined, serum globulin, tuberin, and casein, were each purified as far as possible from other proteins, from multivalent anions and cations, and from all but the last trace of electrolytes, by special methods adapted to the nature of each. They were first prepared either as ammonium or as sodium compounds. Analyses were then made of the amount of ammonia or of sodium in these compounds, and the amount of hydrochloric acid required to neutralize the base and precipitate the protein at its isoelectric point<sup>10</sup> was calculated.

The acid used was usually 0.01  $\times$  hydrochloric acid, and was delivered very slowly from a capillary tip extending well into the solution. The solution was continuously and rapidly mixed by a motor-driven glass screw-shaped stirrer which constantly forced fresh portions of the protein past the glass tip from which the acid was being delivered, in a manner similar to that described by Baker and Van Slyke (37). In this way the protein was never exposed to the denaturing effect of a local excess of acid. After precipitation had begun, the process of neutralization was further retarded in order to allow new states of equilibrium to be fully attained. As the end was approached small samples were removed, and the hydrogen ion concentration electrometrically determined.

The isoelectric reaction was usually reached, at least in the case of serum globulin, before the calculated amount of base had been neutralized. The base in

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The measurements that have been made thus far suggest that when the hydrogen ion concentration of such a protein solution varied it moved in the direction of neutrality.

<sup>10</sup> Loeb has recently discussed the significance of the isoelectric point for the purification of protein (36).

these cases was ammonia, and was determined in the manner described by Sørensen (13), in the filtrate and washings from globulin coagulated by heat at its isoelectric point. It is therefore possible that the very small excess of ammonia reported was due in part, or in whole, to the splitting of ammonia from the protein molecule. That this is involved in the coagulation of proteins by heat is rendered probable by the investigations of Sørensen and Jürgensen (38) on albumins, and by the observation that such proteins as casein and glutenin, which are very slightly soluble at their isoelectric points are not heat-coagulable. The alternative possibility is that serum globulin is capable of combining with a certain amount of base even at its isoelectric point. This will be the subject of another communication. In Table I are collected the ammonia concentrations in the serum globulin preparations that have been studied thus far, and also the hydrochloric acid that was required to bring them to the isoelectric point.

TABLE I.  
*Analyses of Serum Globulin Preparations.*

Preparation of serum globulin.	Fraction.	Protein.	pH <sup>+</sup>	NH <sub>3</sub> in preparation.	HCl used to bring protein to isoelectric point.	Excess ammonia (NH <sub>3</sub> - HCl).	Excess ammonia (NH <sub>3</sub> - HCl) / NH <sub>3</sub>
		gm.		mols	mols	mols	per cent
I*	No fractionation.		7.0	0.0014	0.0010	0.0004	28.6
II*	Pseudoglobulin.		7.0	0.0038	0.0029	0.0009	23.7
IVa	"	34.15	6.9	0.0051	0.0040	0.0011	27.5
IVb	Euglobulin.	14.73	7.4	0.0029	0.0023	0.0006	26.1
Va	Pseudoglobulin.	32.13	7.1	0.0035	0.0025	0.0010	28.6

\* Serum Globulins I and II were prepared in collaboration with Professor Sørensen at the Carlsberg Laboratorium in Copenhagen, early in 1920. They were prepared as ammonium compounds and were not brought to the isoelectric point. The data that are given are derived from certain experiments in which the protein was brought to the isoelectric point in order to study the solvent action of neutral salts. The method of further purifying globulin at its isoelectric point was subsequently devised.

Stirring was always continued for many hours after the isoelectric point was reached, in part to break up the flocks or aggregates that form under these conditions, and to prevent the occlusion of impurities in them, in part to hasten the attainment of equilibrium in so sluggish a system. Occasionally it has been found necessary to break the larger aggregates with a pestle and mortar. The protein was finally returned to the cold room, from which it was never removed for

more than a few hours, and the precipitate allowed to settle. The water-clear supernatant liquid was then decanted; distilled water added approximately to the same volume; and the precipitate again stirred for several hours. This operation was usually repeated from six to fifteen times, and was only stopped when the wash water no longer contained chloride, and when the amount of protein dissolving in the wash water had become constant.

The course of the purification of the euglobulin fraction of preparation IV, as measured by the protein nitrogen in an aliquot of successive washings, is tabulated below. Approximately 14 gm. of this material (14.72 gm. before neutralization) were triturated each day with distilled water. The total volume was 350 cc., and about 190 cc., or slightly more than half of the clear supernatant liquid was decanted each morning. It will be noted that at first the concentration of

TABLE II.  
*Purification of Serum Globulin IV b.*

Wash water No. ....	1	2	3	4	5	6	7
Nitrogen in 10 cc. of wash water, mg.	7.60	3.64	1.86	1.40	0.94	0.80	0.63

soluble protein was also approximately halved each day, but that as the purification proceeded, it approached a constant value.

It is apparent that this method of purification is only applicable to relatively insoluble proteins, since the loss of material would otherwise be prohibitive. In the case of relatively insoluble proteins, however, it has the advantage of ensuring the identity of the product. For unless two proteins have the same isoelectric point, and very nearly the same solubility, this procedure may be used to effect their complete separation. The more soluble protein dissolves in the successive wash waters, and can be recovered from them by concentrating in dialyzers, under negative pressure. It will, however, still contain a trace of the less soluble protein.

Recourse was first had to this method in the preparation of the pseudoglobulin fraction of serum IV. When this fraction was rendered isoelectric a large amount of protein was at first found in the

wash water.<sup>11</sup> This was for the most part globulin like the rest, dissolved by the ammonium chloride formed in the neutralization of the ammonium globulinate by hydrochloric acid. The wash waters containing this protein were accordingly dialyzed further and concentrated.

When the isoelectric protein had finally ceased to give off impurities and readily soluble protein, it was suspended in the desired volume, and aliquot parts used for analyses. It has been found impracticable to dry proteins, since it is almost impossible uniformly to wet their surface after they have been dried by alcohol and ether. As a result, there can be no guarantee that a true equilibrium is subsequently reached between all of the protein and its solvent. Moreover, it has been thought preferable, for the purposes of this investigation, to retain any lipoid that might be in fixed combination with our proteins, rather than risk the danger of denaturing them by alcohol or ether.

### *The Measurement of Solubility.*

In order to obtain reproducible measurements of the solubility of a protein a great many precautions must be observed. To saturate the solution with protein it is necessary to bring the heavy flocculent precipitate into intimate contact with the solvent for a long period of time. This can only be accomplished by protracted mechanical agitation, since the diffusion velocities of the proteins are exceedingly low because of their large molecular weights. If the protein precipitate were not enormously subdivided, and not brought into intimate contact with every part of the solvent, the latter would remain unsaturated.<sup>12</sup> If the ordinary methods of agitation are used, however, the solution foams, its concentration changes, and a portion of the protein is likely to become denatured. Moreover, soft glass containers cannot be used, since in them the protein combines with enough alkali to increase its solubility appreciably.

<sup>11</sup> The isoelectric solubility in my earlier preparations of serum globulin, which had not been washed at the isoelectric point, was as high as 75 per cent of the total protein present.

<sup>12</sup> Under these circumstances the solvent may enter the protein-rich phase, because of its greater diffusion velocity, and give the appearance of swelling.

Our practise has been to pipette an aliquot part of a preparation into a Pyrex volumetric flask of from 25 cc. to 100 cc. capacity. When this was at first attempted it was found that the error due to sampling the suspension of a flocculent protein precipitate was frequently as

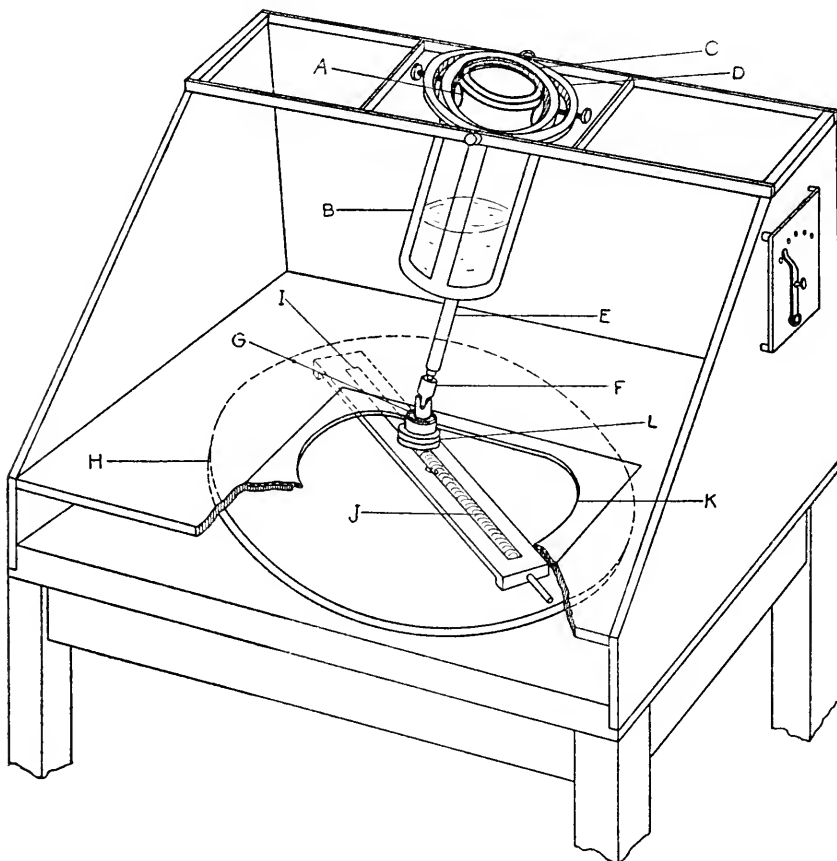


FIG. 1.

great as 3, or even 5 per cent. Accordingly an apparatus was devised by means of which the protein precipitate could be maintained in a state of fine subdivision during pipetting (Fig. 1).

This was accomplished by placing the bottle (A) containing the protein in a carriage (B) hung, as is a compass, in concentric rings (C) and (D). As a result,

the neck of the wide mouthed bottle that is used is always in the same position, and a pipette may be inserted and a sample withdrawn even when the bottle is performing more than a revolution a second. The base of the carriage is attached through a sliding valve (*E*) and a universal joint (*F*) to an eccentric (*G*) on a driving wheel (*H*). Instead of being fixed at a definite distance from the centre of the wheel, (in which case the motion of the bottle would be circular) the eccentric moves freely on ball-bearings in a slot (*I*) but is forced by a spring (*J*) to impinge on a track (*K*) with which it makes contact through a roller-bearing (*L*). The shape of the track alone determines the motion of the carriage. An ovoidal shape has been adopted in order at once to prevent foaming and centrifugal action. With the aid of this apparatus we have been able to sample protein suspensions with an accuracy of 0.3 per cent.

After the protein had been delivered into the volumetric flasks the solvent was slowly added, drop by drop, from a burette until it rose to the graduated mark on the neck of the bottle. In certain experiments, the space above was further reduced by the addition of glass beads of a grade that gave off no measurable alkali, and by a drop of toluene added to prevent bacterial action. The flasks were finally stoppered, fixed in place in the carriage of a specially designed shaking machine (Fig. 2), and shaken in a water thermostat at  $25.0^{\circ} \pm 0.1^{\circ}\text{C}$ . While the shaking machine was in motion, the necks of the flasks were covered with inverted test-tubes to prevent contamination from the spray of the bath.

As the carriage went back and forth on tracks in the water bath, the glass beads in the small volumetric flasks functioned as a very efficient ball mill and kept the protein precipitate mechanically suspended. In our experience the heavy flocculent isoelectric protein has usually been brought into equilibrium with its solvent in from 24 to 72 hours. The shaking machine was then stopped, the contents of the flasks allowed to settle slightly, and the saturated solutions filtered on No. 42 Whatman filter papers. By means of a water jacket through which the water of the thermostat was kept in constant circulation by a rotary pump, the filtration was carried on at nearly the same temperature as the bath. At least 8 cc. of the protein solution was always used to wash the filter paper, and was accordingly discarded. Aliquot parts of the remainder of the filtrate were measured out with carefully calibrated pipettes into Kjeldahl flasks. Analyses were usually made in triplicate. 20 cc. of sulfuric acid

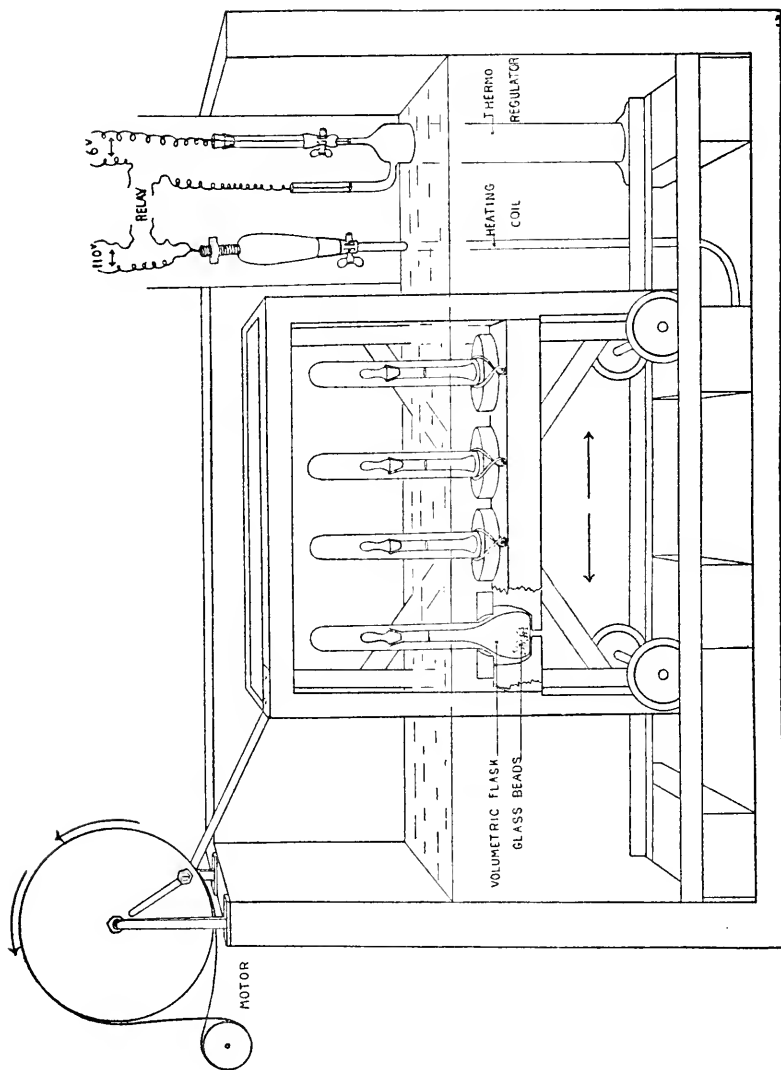


FIG. 2.



(Baker and Adamson: C. P.), 5 gm. of potassium sulfate and 0.7 gm. of mercuric oxide were added, and the protein was digested. When digestion was complete, the colorless solution was cooled and diluted to about 250 cc. with distilled water. 1 gm. of sodium hypophosphite was then added to reduce the mercuric oxide, and 75 cc. of saturated sodium hydroxide were added to neutralize the sulfuric acid and liberate the ammonia. The ammonia was then distilled into a measured amount of  $N/7$  hydrochloric acid. The excess of acid together with a blank containing the same amount of acid were then titrated with  $N/14$  sodium hydroxide, a combination of methylene blue and methyl red being used as indicator (39). Sodium hydroxide of this strength was used, since each cc. required to neutralize the blank in excess of that required to neutralize the unknown then corresponds to 1 mg. of nitrogen, (atomic weight of  $N = 14.01$ ).

#### *The Solubility of Serum Globulin.*

Serum globulin was the first protein that we purified from other proteins, from acids, bases, and salts, and from its own soluble compounds to a sufficient extent to obtain a product of constant solubility,—a product that would dissolve in water to a constant extent. The results of five experiments are recorded in Table III. They are in striking contrast to what they would have been had a second protein, or a soluble form of the same protein, been present. A twentyfold variation in the amount of suspended protein produced no commensurate change in solubility, though there was a very slight increase in solubility with an increase in the amount of the preparation. The solubilities recorded are the average of three analyses. They indicate that serum globulin prepared in this way only dissolves in 1 liter of water at 25°C. to the extent of approximately 0.1 gm.

Serum globulin has now been prepared in this manner a second time, and several further precautions taken both in its purification, and in the determination of its solubility. The globulin in the first preparation (IV) was derived from citrated plasma of the cow, in the second from cow serum. In both preparations the so called pseudoglobulin fraction was used. This fraction is supposedly freer from lipoid (40) and from phosphorus (40, 41) than is euglobulin. Whether

or not these are the same or different proteins we shall consider at another time from the point of view of their respective solubilities.

The pseudoglobulin was freed from euglobulin by repeated fractional precipitation with ammonium sulfate. The euglobulin is supposed to be completely precipitated from a solution that is one-third saturated with respect to ammonium sulfate, and the pseudoglobulin from a solution that is one-half saturated. In practise it was found necessary to adopt the procedure described by Haslam (41). Both precipitates were collected, redissolved, and again one-third saturated with ammonium sulfate. The filtrate from the reprecipitated euglobulin was then added to the pseudoglobulin fraction, and a precipitate appearing in the pseudoglobulin fraction combined with the

TABLE III.  
*Solubility of Serum Globulin IVa.*

T. =  $25.0^{\circ} \pm 0.1^{\circ}\text{C.}$

Experiment No.	Date.	Protein nitrogen suspended in 100 cc. H <sub>2</sub> O at beginning of experiment.				
		mg. 10.9	mg. 21.7	mg. 38.0	mg. 54.3	mg. 190.0
		Protein nitrogen in 25 cc. filtrate.				
6	1921 June 13	mg. 0.37	mg. 0.56	mg. 0.37	mg. 0.56	mg. 0.44
9	" 21					

euglobulin.<sup>13</sup> The pseudoglobulin was reprecipitated thirteen times in preparation IVa, and nine times in preparation V. Serum globulin Va represents a smaller and possibly a better defined fraction. Howe (42) has recently presented additional evidence for the existence of two pseudoglobulin fractions (43, 44, 45); the first precipitated at approximately 0.43 saturated ammonium sulfate. In our experience this fraction represents about three-fourths of all the pseudoglobulin.

The method that has been used in preparing this globulin as an ammonium compound was first worked out in 1919–20 in collaboration with Professor S. P. L. Sørensen, at the Carlsberg Laboratorium. It involves operations of two kinds.

<sup>13</sup> The precipitation of proteins by electrolytes is not independent of the protein concentration.

In the first place freeing the protein from all but one salt, ammonium sulfate. In the second place freeing the protein from the sulfate ion. The first step was accomplished by reprecipitating with ammonium sulfate at different hydrogen ion concentrations. The protein was freed from anions other than the sulfate by reprecipitation with c.p. ammonium sulfate from dilute sulfuric acid solution, approximately  $10^{-4}N$ , and from cations other than ammonia by reprecipitation from solution in dilute ammonia. The globulin was then dialyzed, isosmotically, in the manner described by Sørensen (13). A slight excess of ammonia was maintained in the dialyzers until the globulin was completely freed from sulfate. The transformation of the soluble ammonium-globulin compound into the very slightly soluble uncombined isoelectric globulin has already been described.

A somewhat different and more exact procedure was adopted in studying the isoelectric solubility of serum globulin Va. Not only were different amounts of the globulin preparation used to saturate the same volume of water, but the same precipitate was used to saturate successive additions of water. The dates are recorded on which samples were removed for analysis. On these dates a further amount of fresh water was added and saturation recommenced. The results obtained in this way were far more concordant. They are tabulated in Table IV. As in preparation IV, more protein dissolved in the flasks which contained most protein. That this was for the most part due to the fact that all of the soluble protein had not been removed, is suggested by the fact that solubility soon fell to a lower and constant value in the flasks containing the three smallest amounts of globulin.

The persistent higher solubility in the flasks containing most precipitate is probably to be ascribed to another phenomenon that need not be considered at this time.

Twenty-four nitrogen determinations, made upon the filtrates of solutions saturated with three different amounts of protein, on 4 different days, after their solubility had become constant, agree within the errors of measurement. The average of these determinations is 0.30 mg. protein nitrogen in 25 cc., or 12.0 mg. protein nitrogen in 1 liter. This result is not very different from the lower results obtained with preparation IV. If we adopt 15.85 as the percentage nitrogen in this protein the most probable solubility of the pseudoglobulin fraction of serum becomes 0.07 gm. per liter at 25°C.

Although so insoluble when uncombined it should be noted that 1 gm. of this preparation was completely dissolved by  $1 \times 10^{-4}$  mols. of sodium hydroxide, or by slightly less than that amount of hydrochloric acid. The compounds of serum globulin with acids and with bases are very soluble indeed. The smallest trace of a soluble compound would have appreciably increased solubility. The measure-

TABLE IV.  
*Solubility of Serum Globulin Va.*  
T. =  $25.0^\circ \pm 0.1^\circ\text{C}$ .

Experiment No.	Date.	Protein nitrogen suspended in 100 cc. H <sub>2</sub> O at beginning of experiment.			
		mg. 35.27	mg. 70.55	mg. 176.37	mg. 352.75
		Protein nitrogen in 25 cc. filtrate.			
		mg.	mg.	mg.	mg.
108	1922				
	Apr. 24	0.29	[0.52]	[0.72]	
		0.32	0.52	0.70	
		0.29	[0.50]		
	" 26	0.32	0.28	0.28	[0.62]
		0.29	0.29	0.33	0.62]
		0.32	0.27	0.31	
	May 1	0.30	0.31	0.31	[0.49]
		0.30	0.32	0.35	0.52]
		0.29	0.29	0.31	[0.51]
	" 3			0.31	[0.51]
				0.29	0.52]
				0.31	[0.51]
	Average.....	0.30	0.29	0.31	

ments reported must therefore have been upon the pure ampholyte, upon the undissociated molecule and its dissociated ions.

Certain proteins retain the power to dissolve in acid and alkaline solutions even after becoming denatured. Denatured globulin will not, however, dissolve in the solution of a neutral salt. No trace of denatured material was present in this preparation. It readily dissolved to a water-clear liquid in 0.1 N solution of sodium chloride.

Although solubility data suggested that no appreciable free electrolyte was present in these preparations, their ash contents were considerable. The ash constituted 0.7 per cent of preparation IV. In preparation V more careful methods of purification and precipitation reduced the ash to 0.3 per cent.

*The Solubility of Tuberin.*

The measurements upon tuberin, although they have been made on but one preparation, and although they are not nearly so accurate

TABLE V.  
*Solubility of Tuberin I.*

T. =  $25.0^{\circ} \pm 0.1^{\circ}\text{C}$ .

Experiment No.	Date.	Protein nitrogen suspended in 100 cc. H <sub>2</sub> O at beginning of experiment.		
		mg. 3.05	mg. 15.25	mg. 30.50
		Protein nitrogen in 25 cc. filtrate.		
204	1922	mg.	mg.	mg.
	Jan. 25	0.44		
	" 30	0.37	0.55	
	Feb. 9		0.55	
206	" 15			0.57*
	" 21			0.66*

\* 5 cc. sample used.

as those that have been presented for serum globulin, or those that will be recorded for casein, are included for two reasons. In the first place because they establish the approximate constancy of solubility of another globulin derived from an entirely different source; in the second place because a study of "the relation between the isoelectric point of a globulin (tuberin) and its solubility and acid combining capacity in salt solution" (14) was responsible for the inception of these studies. The data obtained at that time showed the effect of even a uni-univalent salt, sodium chloride, in shifting the minimum of protein solubility. This phenomenon has already been discussed.

They were not correctly interpreted, however, since the isoelectric point was inferred to occur at a more acid reaction than now seems probable from observations of cataphoresis (46). The inapplicability of cataphoresis in determining the isoelectric point of a globulin has also been discussed.

The same methods were used in purifying tuberin after it had been extracted from the potato, and in determining its solubility, as were used with serum globulin. Tuberin has been found to be only slightly more soluble than the latter. The measurements in the three experiments recorded in Table V indicate a solubility in water of approximately 0.1 gm. in 1 liter at 25°C. Had a larger amount of material been available, and a more protracted experiment been possible, a slightly smaller value might have been obtained.

#### *The Solubility of Casein.*

Casein has previously been prepared in a state of great purity by a method very similar to the one that we have described (37). Baker and Van Slyke were able to show that if casein was very carefully precipitated at its isoelectric point, and was then triturated with distilled water, a product of very low ash could be obtained. We have used a modification of this method in the early stages of the purification. This modification depends upon the observation of Loeb (47) that if a divalent rather than a monovalent base is used to dissolve isoelectric casein, twice the normal concentration is required. As a result the hydroxyl ion concentration of casein dissolved by calcium hydroxide is very much greater than that dissolved by sodium hydroxide. According to Loeb the pH of the soluble sodium compound of casein is 7.02 and of the soluble calcium compound 10.53. Presumably divalent bases of the type of calcium hydroxide form insoluble acid salts with casein at neutral reactions.

Our practise has rested upon this phenomenon. After the casein has been precipitated at its isoelectric point and washed with distilled water according to the method of Baker and Van Slyke, only enough sodium hydroxide was added to bring the casein to a neutral reaction. In this our method differed from that of Hammarsten (48) in which enough sodium hydroxide is added to dissolve completely the casein.

Loeb's observation suggests that the casein that persists at a neutral reaction is largely combined with divalent bases. We have accordingly removed and discarded this precipitate, either by centrifugation (in a Sharpless centrifuge at nearly 30,000 revolutions a minute) or by filtration through filter paper pulp. The casein in the filtrate or in the centrifugate was then reprecipitated at its isoelectric point by the addition of hydrochloric acid, washed, and again dissolved by sodium hydroxide. It has been our experience that the casein completely dissolved the second time at a neutral reaction. The casein in this clear neutral solution was finally precipitated and purified at the isoelectric point in the manner that has already been described.

Casein has generally been supposed to be nearly completely insoluble. Laqueur and Sackur (49), it is true, observed that a small amount of their casein preparations always dissolved in water, but decided that this amount was negligible. Our experiments upon different preparations show that casein is no less soluble than the globulins that have thus far been prepared in states of comparable purity.

As in the cases of serum globulin and tuberin, the first measurements of the solubility of precipitated casein were high. In this case also this can be explained by assuming that casein forms readily soluble compounds. The nature of these compounds and the extent to which they can exist in the neighborhood of the isoelectric point will be considered in another communication. The solubility of two preparations of uncombined casein has been determined. The results of thirty-two nitrogen determinations made upon the casein dissolved in five different flasks, containing different amounts of precipitate, on 3 different days, agree within the errors of measurement. Since the earlier work, though not nearly so accurate, is in fair agreement with these later determinations, the average may be taken as the most probable solubility of casein, and yields the result that 0.11 gm. of casein dissolves in 1 liter of water at 25°C.

Many of the measurements that are reported were made by Miss Jessie L. Hendry. It gives me pleasure to express my indebtedness for her invaluable aid.

TABLE VI.  
*Solubility of Casein III.*

T. = 25.0° ± 0.1°C.

Experiment No.	Date.	Protein nitrogen suspended in 100 cc. H <sub>2</sub> O at beginning of experiment.	
		mg. 4.0	mg. 20.0
		Protein nitrogen in 25 cc. filtrate.	
33	1921	mg.	mg.
	Nov. 29	0.36	
	Dec. 6	0.38	0.35
	1922		
	Jan. 31		0.48
37	Feb. 5		0.49
	" 14	0.57	
	" 22	0.57	
	" 24		0.59

TABLE VII.  
*Solubility of Casein V.*

T. = 25.0° ± 0.1°C.

Experiment No.	Date.	Protein nitrogen suspended in 100 cc. H <sub>2</sub> O at beginning of experiment.				
		mg. 8.55	mg. 17.10	mg. 42.75	mg. 85.50	mg. 171.0
		Protein nitrogen in 25 cc. filtrate.				
39	1922	mg.	mg.	mg.	mg.	mg.
	Apr. 3	0.47	0.42	0.47	0.48	[ 0.72 ]
		0.42	0.37	0.47	0.50	0.92
		0.42	0.47	0.50	0.47	[ 0.72 ]
	“ 11	0.50	0.40	0.50	0.45	0.50
		0.35	0.36	0.45	0.45	0.51
		0.40	0.55	0.45		0.50
	“ 14			0.40	0.40	0.40
				0.40	0.50	0.40
				0.40	0.45	0.45
	Average.....		0.43	0.43	0.45	0.46



## SUMMARY.

1. Two proteins of the globulin type, serum globulin and tuberin, and the protein of milk, casein, have been purified (*a*) of the other proteins and (*b*) of the inorganic electrolytes with which they exist in nature. The methods that were employed are described.

2. All three proteins were found to be only very slightly soluble in water in the pure uncombined state. The solubility of each was accurately measured at  $25.0^{\circ} \pm 0.1^{\circ}\text{C}$ . The most probable solubility of the pseudoglobulin of serum was found to be 0.07 gm. in 1 liter; of tuberin 0.1 gm. and of casein 0.11 gm. The methods that were employed in their determination are described.

3. Each protein investigated dissolved in water to a constant and characteristic extent when the amount of protein precipitate with which the solution was in heterogeneous equilibrium was varied within wide limits. The solubility of a pure protein is therefore proposed as a fundamental physicochemical constant, which may be used in identifying and in classifying proteins.

4. The concentration of protein dissolved must be the sum of the concentration of the undissociated protein molecule which is in heterogeneous equilibrium with the protein precipitate, and of the concentration of the dissociated protein ions.

5. The dissociated ions of the dissolved protein give a hydrogen ion concentration to water that is also a characteristic of each protein.

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## CELL PENETRATION BY ACIDS.

### V. NOTE ON THE ESTIMATION OF PERMEABILITY CHANGES.

By W. J. CROZIER.

(Contributions from the Bermuda Biological Station for Research, No. 139, and from the Zoological Laboratory, Rutgers College, New Brunswick.)

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#### 1. *Effects of Tension.*

In view of the fact that qualitative studies regarding permeability have sometimes been based upon experiments involving intense muscular activity in the tissue examined, I thought it worth while to measure the effect of stretching upon the rate with which acids penetrate the indicator-containing tissue of the nudibranch *Chromodoris* (cf. Crozier, 1916 a).

A piece of the mantle fold, about 2 cm. in length by 0.5 cm. in breadth, was freshly secured by cutting from an individual sufficiently large to supply several such pieces. The piece was then adjusted in a holder, sketched in Fig. 1. This was accomplished by tying to either end a short ligature of waxed thread. One of the ligatures was made fast at *a*, while the other, longer, one was passed over the groove *c*. The longer thread carried at its free end a mass which in the different tests was varied from 5 to 50 gm. The tissue, being fixed in this manner, was lowered into the trough, *A*, containing an acid solution. The time required for the internal color change to be brought about was measured with a stop-watch, while the first evidence of pigment loss could be accurately recognized by watching the edge of the tissue through a horizontal microscope. A slip of milk-glass, mounted back of the tissue, increased the precision of the observations.

By employing a strip of the mantle about 2 cm. in length, there was secured an area in the center of the strip which was free from complications due to the presence of tight ligatures at either end. This was checked by experiments in which the tissue, with the threads made fast, was allowed to hang freely without any attached weights.



From a number of tests of this kind, data were secured for the construction of Fig. 2. It is apparent that even moderate tension exerts a very decided effect upon the penetrability of this tissue for 0.01 N dichloroacetic acid. A similar result was had with other weak acids; the coagulative action of the stronger acids tends to obscure the phenomenon.

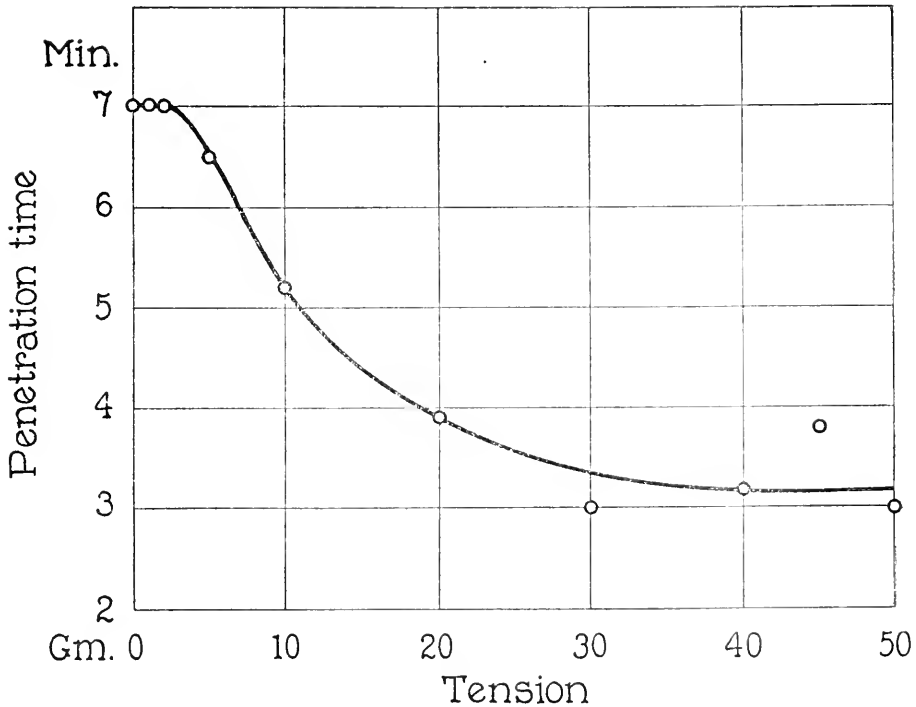


FIG. 2. Penetration of tissues under tension, by 0.01 N dichloroacetic acid; 25°C. Ordinate, penetration time, minutes; abscissa, tension, gm.

Since pieces of mantle tissue subjected for 10 minutes to tensions of 30 to 40 gm., in sea water, quickly regained about their normal penetrability when the traction was removed, the effect of tension is (within limits) reversible.

The effect of tension noted in these experiments cannot be ascribed to manipulation of the tissue during its preparation. Undue

exposure to the air has a very marked influence in the direction of *decreasing* its penetrability. The penetrability of isolated fragments of the *Chromodoris* mantle kept in sea water remained very nearly constant for some 24 hours after their removal.

The influence of tension is equally apparent in relation to diffusion of pigment from the mantle tissue. In one set of experiments, which may be cited as typical, the following observations were recorded:

*Experiment 9.26b.*—Mantle tissue from two nudibranchs 8.5 cm. long; 0.01 N dichloroacetic acid; 25°C.

Animal.	Stretching mass.	Penetration time.	First sign of pigment loss.
	<i>gm.</i>	<i>min.</i>	<i>min.</i>
A	0	7.0	15.0
	10	5.5	10.0
	20	3.7	4.0
B	0	7.1	12.0
	30	3.2	4.0
	50	3.0	2.5

The observations on the outward diffusion of pigment are difficult to summarize, because it is necessary to choose for comparison individuals with closely agreeing pigmentations. There was no doubt of the qualitative result, however, which was clearly that the rate of spontaneous pigment diffusion, in acid, is greater when the tissue is stretched. Moreover, the effect of tension was usually greater with respect to pigment diffusion than in relation to penetration of acid (as evidenced by color change). The outcome of these tests may be held to indicate that under tension the resistance of a protoplasmic surface to the diffusion of electrolytes undergoes a (more or less reversible) decrease.<sup>1</sup> The experiments may then have an interesting connection with Carlson's (1907) finding in the

<sup>1</sup> What rôle may be occupied by an actual thinning-out of the cells of the tissue stretched in the present experiments, it is difficult to decide; my chief purpose is to point out, merely, the complication introduced into "permeability" studies by the presence of tractive forces.

*Limulus* heart, where even moderate traction applied to a cardiac ganglion was seen to increase the intensity of nervous discharge as reflected in the heart beat. And it is obvious that in experiments concerning permeability (*cf.* Lillie, 1909) the presence or absence of relatively intense muscular activity, itself employed as an index of excitation, may introduce a complication difficult to evaluate.

## 2. Effects of Stimulation.

Pieces of the mantle of the nudibranch were cut from individuals of medium size. The fragments excised were about 1.5 cm. long. Non-polarizable electrodes, 4 mm. apart, were applied to the pigmented surface. Current was obtained from a Harvard inductorium with two Leclanché cells in parallel in the primary circuit, the secondary coil being at "5." The stimulation was purposely made rather severe. The mantle fragments became contracted when stimulated, since they contained smooth muscle fibres. Considerable quantities of slimy secretions were expelled from the stimulated surface. After being stimulated the piece of tissue was immersed in an acid solution, and the penetration time was compared with that exhibited for a control piece previously unstimulated.

*Experiment 8.33.2.*—Four pieces of tissue, each stimulated for 30 seconds, and four control pieces, unstimulated; 0.10 N HNO<sub>3</sub>; 21°C.

	Penetration time.	
	Unstimulated.	Stimulated.
	<i>min.</i>	<i>min.</i>
(a)	6.5	3.2
(b)	6.0	2.5
(c)	6.0	4.0
(d)	6.5	5.0
Mean.	6.3	3.7

The pronounced increase of penetrability demonstrated in such tests was found to hold for other acids as well.

The increase of penetrability is somewhat augmented according to the duration of the stimulation.

*Experiment 8.33.3.*—Individual A, 6 cm. long; B, 11 cm. long; 0.05 N HNO<sub>3</sub>; 20°C.

Tissue from animal.	Stimulation.	Penetration time.	
		Unstimulated.	Stimulated.
	<i>min.</i>	<i>min.</i>	<i>min.</i>
A	0.5	2.45	1.5
	0.5	2.5	1.3
	1.0	2.5	0.9
B	0.5	5.5	5.5
	1.0	5.5	3.7
	1.0	5.5	3.0
	1.5	5.6	3.7

This effect can hardly be referred to the contraction of the tissue; the mantle fragments relaxed when the electrodes were removed, before being placed in acid; and they always contracted upon immersion in acid, whether previously stimulated or not. Nor can the extruded mucus be directly involved, for the same reason. Moreover, as pointed out beyond, mucus extrusion also occurs under the influence of chloroform, which has an opposite effect upon penetrability.

When a small spot upon a fair sized piece of the nudibranch integument was stimulated, and the whole then placed in acid, the increased penetrability was manifest merely upon the immediate site of excitation. Thus, in one experiment with 0.05 N HNO<sub>3</sub>, a stimulated area turned pink in 2.45 minutes, while the general surface of the tissue did not do so until 3.20 minutes had passed, in spite of the fact that mucus discharge had occurred over the entire surface.

The method of observation is such that the results may not be interpreted in terms of "permeability," since the intracellular color change serving as an index of acid penetration is dependent upon the relation of the pigment to the rest of the cell contents; this relation might be altered by excitation of the epithelium. Nevertheless, it is entirely probable that in these tests the penetrability of the protoplasm toward acid is markedly increased as the result of faradic stimulation.



### 3. *Anesthetics.*

Ether, ethyl alcohol, chloroform, and  $\text{MgSO}_4$  all produce a very decided increase in the apparent time of penetration of acids.

*Experiment 8.54.1.*—Tissue from several animals of the same size; acid, 0.05 N HCl. Pieces of mantle immersed for the intervals stated in sea water containing M ethyl alcohol, then transferred to acid.

Tissue from animal.	Time in alcohol solution.	Penetration time.	
		Alcohol.	Control.
	<i>min.</i>	<i>min.</i>	<i>min.</i>
A	3	5.2	5.0
	3	7.0	5.0
	10	10.0	5.0
	20	10.0	
	20	9.1	
B	2	3.5	2.5
	10	5.0	2.6
	20	9.1	

A number of tests showed essentially similar conditions under the action of other anesthetics. The primary effect of these materials is to decrease the penetrability of the tissue toward acids. Ether, one-half saturated in sea water, and chloroform, one-fifth saturated, decreased the apparent penetration rate of even 0.1 N HCl by 50 per cent, in 2 minutes and in 5 minutes respectively.

### 4. *Pigment Diffusion.*

The outward diffusion of intracellular coloring matters has frequently been employed as a criterion of increased permeability to the pigment involved, and by inference to other substances also. In addition to other sources of error, this method of observation tends to ignore the fact that the pigment concerned may be held in the cell, not by the state of the cell surface primarily, but by the relation of the pigment, especially when in the form of droplets, to the cytosome as a whole.

As bearing upon the value of pigment diffusion as an index of permeability increase, I would cite experiments showing that the

penetrability of the mantle epithelium of *Chromodoris* toward acids may be caused to vary in the direction either of increase, or of diminution, while the spontaneous diffusion of pigment (uncomplicated by muscular contractions, in this instance,) simultaneously changes in a directly contrary manner. The pigment involved serves as indicator of internal acidity and is at the same time the freely water-soluble coloring matter concerned in visible outward diffusion. For comparative tests it is necessary to use tissues colored in precisely the same way by this pigment.

	<i>min.</i>
<i>Experiment 8.34.3.</i> —(a) Two pieces of mantle placed in 0.1 N HCl; penetration time (24°).....	2.16
	2.05
(b) Two similar pieces placed in sea water one-half saturated with $\text{CHCl}_3$ , for 5 minutes; then put into 0.1 N HCl; penetration time (24°).....	2.25
	3.75

In (a) loss of pigment was evident in 4 and 10 minutes, respectively; in (b), within 2 minutes in both cases.

*Experiment 8.42.1.*—Four pieces of mantle tissue from a single individual were used; two were placed in 0.1 N  $\text{HNO}_3$ , the penetration times being 3.25 and 3.50 minutes, respectively; two others were placed for 10 minutes in sea water containing 0.1 M urea, then transferred to 0.1 N  $\text{HNO}_3$ —the penetration times being now 0.5 and 0.3 minutes. In the first case pigment diffusion was observed in 7 minutes, in the second in 9 minutes.

These tests, typical of a number made, show clearly that the condition of the tissue used may differ markedly when regarded from the standpoints (1) of penetrability for acids, and (2) of the readiness with which spontaneous loss of pigment occurs. A long series of observations upon the penetration of various acids showed, in addition, that practically no correspondence obtains between the relative ease of penetration and the speed with which pigment is lost to the surrounding solution. With mineral acids, pigment loss in general followed the internal color change indicative of penetration; while with weak acids, and especially with fatty acids, even in the case of relatively concentrated solutions penetrating with high velocity, loss of pigment usually preceded the internal sign of acid penetration. Quite apart from the fact that it seems inadvisable to speak of “permeability” as a general property, having reference indis-

criminally to all classes of substances, the facts already enumerated make it hazardous to lay much weight upon pigment diffusion as an index of permeability changes (Crozier, 1916 *b*). Thus in Lillie's extensive operations with *Arenicola* larvæ (1913), the fact that in the presence of anesthetics pigment diffusion is retarded, has been urged as evidence for the restraint by anesthetics of permeability-increasing agencies; on the other hand, when mantle tissue of *Chromodoris* is subjected to the action of anesthetics, pigment is lost much more readily than otherwise, even though the resistance of the tissue toward the diffusion of acids is by this action markedly enhanced.

#### SUMMARY.

The penetration of acid into mantle tissue of *Chromodoris zebra* is accelerated after local faradic stimulation, and is retarded by brief treatment with anesthetic solutions. The spontaneous outward diffusion of intracellular pigment is an inadequate criterion of "permeability." Outward diffusion of pigment and penetration of acid are both facilitated when the tissue is artificially put under tension.

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# THE COMBINATION OF GELATIN WITH HYDROCHLORIC ACID.

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Titration curves for gelatin with different acids have been obtained by Loeb,<sup>1,2</sup> and used to show that the protein reacts stoichiometrically with the different acids. The amount of a strong acid combined with the protein may be obtained from the titration curve (in which the abscissæ are pH values and the ordinates concentrations of acid) by subtracting from the ordinates the amounts of acid necessary to bring the same volume of water, without protein, to the same pH values. In this way Loeb<sup>2</sup> obtained a curve for the amount of HCl combined with 1 gm. of gelatin at different pH values. Similar curves were obtained by Tague<sup>3</sup> for the combination of amino-acids with NaOH, and by the writer<sup>4</sup> for the combination of edestin with acids. Each of these combination curves appeared to become horizontal beyond a certain pH; *i.e.*, after enough acid (or alkali, in the case of Tague's experiments) had been added, the amount combined with the ampholyte became constant.

In a recent paper by Lloyd and Mayes<sup>5</sup> a curve is given to represent the amount of HCl combined with 1 gm. of gelatin. This curve, however, does not become horizontal, but rises with increasing acidity in a rather discontinuous manner. These authors did not obtain the amount of combined HCl in the way just described, but calculated it by the formula  $N' = N - \frac{[H^+] \text{ corr.}}{\alpha}$  where  $[H^+] \text{ corr.} = \sqrt{[H^+] \times [Cl^-]}$ ,

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1920-21, iii, 100.

<sup>2</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 50-51.

<sup>3</sup> Tague, E. L., *J. Am. Chem. Soc.*, 1920, xlii, 173.

<sup>4</sup> Hitchcock, D. I., *J. Gen. Physiol.*, 1921-22, iv, 597.

<sup>5</sup> Lloyd, D. J., and Mayes, C., *Proc. Roy. Soc., London, Series B*, 1922, xciii, 69.

$N$  = normality of total HCl,  $\alpha$  its degree of ionization, and  $N'$  = normality of combined HCl. The values which they used for the degree of ionization were conductivity ratios given by Lewis.<sup>6</sup> Inasmuch as the results of Lloyd and Mayes differed from those obtained by Loeb, it seemed worth while to repeat and amplify his experiments.

Solutions were made up containing 1, 2.5, and 5 gm. of gelatin in 100 cc. of HCl of various concentrations. The gelatin was taken from a stock solution of isoelectric gelatin which had been rendered practically ash-free in the way described by Loeb,<sup>7</sup> and was diluted to twice the concentration required for each set of experiments. 25 cc. samples of these solutions were measured out at 33°C. by a pipette, and each sample was diluted to 50 cc. by the addition of the proper amounts of 0.1 M or 1.0 M HCl and water from burettes. The concentration of the gelatin was checked by dry weight determinations of the amount of gelatin delivered by the 25 cc. pipette, and was accurate to about 1 part in 200. The concentration of the acid used was determined by titration against pure  $\text{Na}_2\text{CO}_3$ , and was accurate to 1 part in 500 or better. The pH of the solutions was determined at 33° with the hydrogen electrode and potentiometer, using rocking cells of the Clark type connected by a salt bridge of saturated KCl to a saturated KCl calomel cell. The pH values are referred to 0.1000 M HCl as a standard, its pH being taken as 1.036 at 33°. The E.M.F. readings obtained were reproducible to within 1 millivolt, which corresponds to about 0.02 pH.

The titration curves obtained in this way are given in Fig. 1, the abscissæ being pH values, and the ordinates total concentration of HCl expressed in millimoles per liter, which is the same as cc. of 0.1 M acid per 100 cc. The curve for 1 per cent gelatin represents three experiments, one of which was performed by Mr. M. Kunitz.

In order to determine how much of the total HCl was not combined with the gelatin, a series of solutions containing only HCl and water was prepared, and the pH values were determined. The values are given in Table I.

<sup>6</sup> Lewis, W. C. McC., *A system of physical chemistry*, London and New York, 3rd edition, 1920, ii, 219.

<sup>7</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 39; *J. Am. Chem. Soc.*, 1922, xlv, 213; *Proteins and the theory of colloidal behavior*, New York and London, 1922, 35.

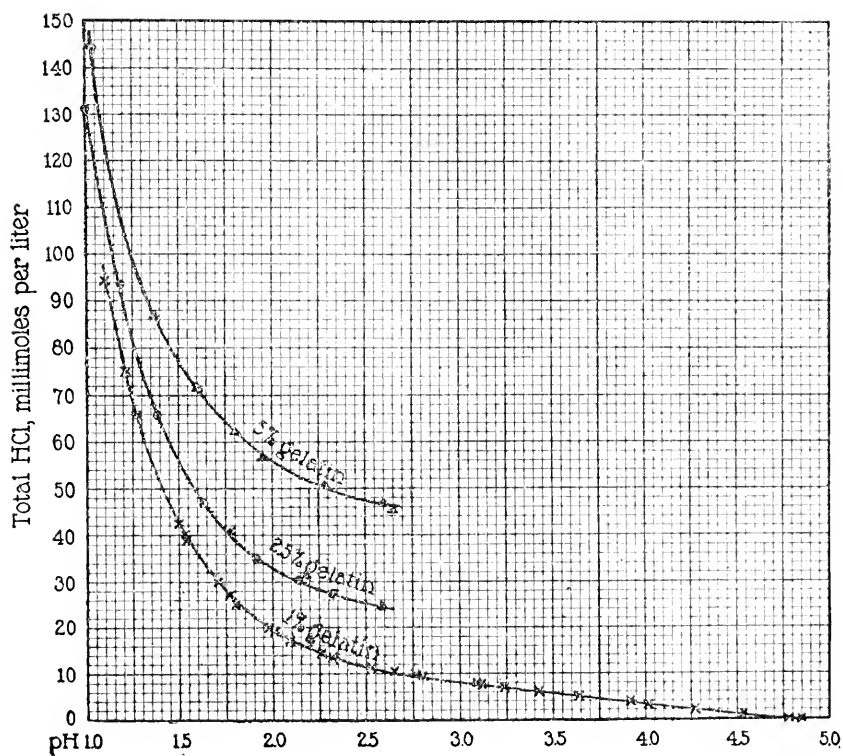


FIG. 1.

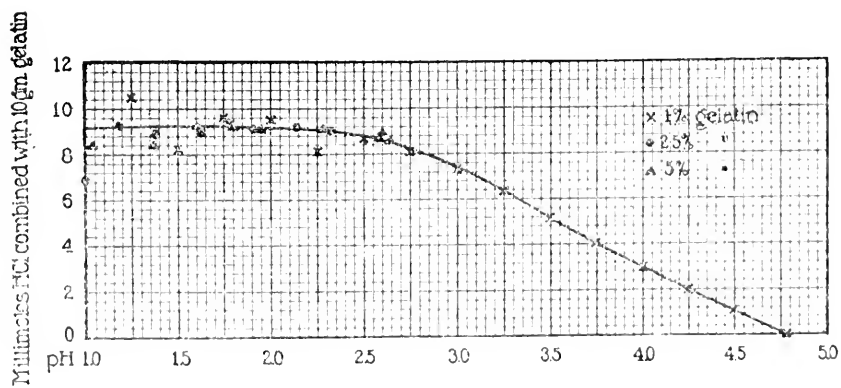


FIG. 2.

TABLE I.  
*Titration of Water with Hydrochloric Acid at 33°C.*

Concentration of HCl, per liter,	0.05	0.10	0.19	0.39	0.68	0.97	1.95	3.89	8.75	15.56	24.31	48.63	100.00	117.7
millimoles. ....	4.35	4.07	3.74	3.38	3.17	3.00	2.70	2.41	2.06	1.81	1.63	1.33	1.04	0.99
pH.....														

TABLE II.  
*Titration of Gelatin with Hydrochloric Acid.*

pH of 1 per cent gelatin chloride*.....	1.25	1.50	1.75	2.00	2.25	2.50	2.75	3.00	3.25	3.50	3.75	4.00	4.25	4.50
Total HCl per liter, millimoles.....	69.5	41.3	27.6	19.5	14.7	11.8	9.8	8.3	6.8	5.5	4.3	3.1	2.0	1.1
Uncombined HCl.....	59.0	33.1	18.0	10.0	6.6	3.1	1.7	1.0	0.5	0.3	0.2	0.1	0.0	0.0
Combined HCl per 10 gm. gelatin, millimoles.....	10.5	8.2	9.6	9.5	8.1	8.7	8.1	7.3	6.3	5.2	4.1	3.0	2.0	1.1
pH of 2.5 per cent gelatin chloride.....	1.00	1.18	1.38	1.63	1.78	1.91	2.14	2.32	2.58					
Total HCl per liter, millimoles.....	131.3	93.8	65.7	46.9	40.8	35.0	30.2	27.2	24.3					
Uncombined HCl.....	114.0	70.5	43.5	24.3	16.7	12.3	7.3	4.8	2.6					
Combined HCl per 25 gm. gelatin, millimoles.....	17.3	23.3	22.2	22.6	24.1	22.7	22.9	22.4	21.7					
Combined HCl per 10 gm. gelatin, millimoles.....	6.9	9.3	8.9	9.0	9.5	9.1	9.2	9.0	8.7					
pH of 5 per cent gelatin chloride.....	1.03	1.37	1.61	1.80	1.95	2.28	2.60	2.64						
Total HCl per liter, millimoles.....	145.0	86.6	71.5	62.1	56.5	50.8	47.1	45.2						
Uncombined HCl.....	103.0	44.4	25.5	15.9	11.2	5.3	2.5	2.3						
Combined HCl per 50 gm. gelatin, millimoles.....	42.0	42.2	46.0	46.2	45.3	45.5	44.6	42.9						
Combined HCl per 10 gm. gelatin, millimoles.....	8.4	8.4	9.2	9.2	9.1	9.1	8.9	8.6						

\* The figures for 1 per cent gelatin chloride were obtained from the curve averaging three experiments.



These results were plotted on a large scale and a smooth curve was drawn through the points. From this curve were read off the concentrations of free HCl present at the pH actually measured in each gelatin chloride solution, and these values were subtracted from the total HCl concentrations of the respective gelatin chloride solutions. The differences accordingly represent the millimoles of HCl combined with the amount of gelatin present in 1 liter, or the cc. of 0.1 M HCl combined with the gelatin present in 100 cc. These values were divided by the number of grams of gelatin in 100 cc. to get the cc. of 0.1 M HCl combined with 1 gm. of gelatin, which is the same as the number of millimoles of HCl combined with 10 gm. of gelatin. Table II indicates the method of calculation, and the final results are plotted in Fig. 2.

It will be noticed that the points lie fairly close to a smooth curve, except in the most acid region, where a small error in the pH may lead to a large error in the difference between the ordinates of two steep curves. The curve is horizontal between pH 1 and 2, indicating that here the gelatin is all combined with the acid. There is no evidence of the discontinuous sections found in the curve of Lloyd and Mayes.<sup>5</sup> This difference is due in part to differences in the experimental curves, but is also due largely to the method of calculation of the combined acid. The method used by Lloyd and Mayes involves the assumption that the uncombined HCl is ionized to the extent indicated by the conductivity ratio for a different concentration of HCl; namely, the concentration of the total HCl present. Moreover, these authors have neglected the difference between the conductivity ratios and the activity coefficients or hydrogen electrode values for HCl, which is clearly brought out by the table given by Lewis<sup>6</sup> from which they obtained their ionization values. The method of calculation used by Loeb, Tague, and the present writer involves the assumption that the same concentration of uncombined HCl is necessary to furnish the same hydrogen ion concentration, as determined by the hydrogen electrode, whether or not gelatin is present. The latter assumption seems to lead to more reasonable results.

The maximum height of the curve in Fig. 2, 9.2 millimoles of HCl for 10 gm. of gelatin, indicates that a 1 per cent gelatin solution has a

normality of 0.0092 with respect to its combination with HCl, or that the combining weight of gelatin is  $\frac{1.0}{0.0092}$ , or about 1,090. While the exact height of the maximum is still more or less uncertain, it is probable that this value of the combining weight is more nearly correct than the smaller values given by Procter,<sup>8</sup> Wilson,<sup>9</sup> Wintgen and Krüger,<sup>10</sup> and Wintgen and Vogel,<sup>11</sup> because the calculation involves simpler and more probable assumptions. Moreover, the earlier workers did not have ash-free or isoelectric gelatin at their disposal.

It is possible to calculate from these results an ionization constant for gelatin, assuming it to react as a mono-acid base. The simplest way of doing this is based on the resemblance between the combination curve and the dissociation curve of a base. The equation for the ionization of such a base is  $\alpha = \frac{k_b}{k_b + [\text{OH}^-]}$  where  $\alpha$  represents the fraction ionized and  $k_b$  the ionization constant. Since the ion product of water,  $k_w$ , is equal to  $[\text{H}^+] \times [\text{OH}^-]$ , this becomes

$$\alpha = \frac{[\text{H}^+]}{[\text{H}^+] + K}, \text{ if } K = \frac{k_w}{k_b}$$

Michaelis<sup>12</sup> has pointed out that if  $\alpha$  is plotted against  $\log [\text{H}^+]$ , at the point where  $\alpha = \frac{1}{2}$ ,  $\log [\text{H}^+] = \log K$ . Applying this to Fig. 2,  $\alpha = \frac{1}{2}$  where the ordinate = 4.6 or  $\text{pH} = \text{pK} = 3.625$ . Accordingly  $K = 2.4 \times 10^{-4}$ . This is of the same order as the value obtained by Procter and Wilson<sup>13</sup> and is intermediate between the values obtained by Wintgen and Krüger<sup>10</sup> and Wintgen and Vogel,<sup>11</sup> but differs by a whole power of 10 from that obtained by Lloyd and Mayes.<sup>5</sup> Inasmuch as the use of this constant leads to only rough agreement with the combination curve in Fig. 2, it is not desired to lay any stress on this calculation. It is quite certain that gelatin is not a mono-acid base, though its combination curve may resemble the ionization curve of such a base.

<sup>8</sup> Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313.

<sup>9</sup> Wilson, J. A., *J. Am. Leather Chem. Assn.*, 1917, xii, 108.

<sup>10</sup> Wintgen, R., and Krüger, K., *Kolloid-Z.*, 1921, xxviii, 81.

<sup>11</sup> Wintgen, R., and Vogel, H., *Kolloid-Z.*, 1922, xxx, 45.

<sup>12</sup> Michaelis, L., *Die Wasserstoffionenkonzentration*, Berlin, 1914, 20.

<sup>13</sup> Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

## SUMMARY.

The amount of HCl combined with a given weight of gelatin has been determined by hydrogen electrode measurements in 1 per cent, 2.5 per cent, and 5 per cent solutions of gelatin in HCl of various concentrations, by correcting for the amount of HCl necessary to give the same pH to an equal volume of water without protein. The curve so obtained indicates that the amount of HCl combined with 1 gm. of gelatin is constant between pH 1 and 2, being about 0.00092 moles.

The writer wishes to express his gratitude for the advice of Dr. Jacques Loeb, under whose direction this work was done.



# THE MECHANISM BY WHICH TRIVALENT AND TETRAVALENT IONS PRODUCE AN ELECTRICAL CHARGE ON ISOELECTRIC PROTEIN.

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## I.

Experiments on anomalous osmosis recently published<sup>1</sup> suggest that isoelectric gelatin assumes a negative charge when a solution of a salt with tetravalent anion, *e.g.*  $\text{Na}_4\text{Fe}(\text{CN})_6$ , is added; and that isoelectric gelatin assumes a positive charge when a salt with a trivalent cation, *e.g.*  $\text{LaCl}_3$ , is added. In these experiments special care was taken that the pH of the solutions was that of the isoelectric point of gelatin; *i.e.*, pH 4.7.  $\text{CaCl}_2$ ,  $\text{Na}_2\text{SO}_4$ , and  $\text{NaCl}$  had apparently no such effect on the charge of isoelectric gelatin.

The question arose whether it was possible to support this suggestion by direct measurements of the p.D. between isoelectric solid gelatin and the surrounding salt solution of pH 4.7; and if this was the case what was the reason for this peculiar action of trivalent and tetravalent ions.

The method of the experiments was as follows: Doses of 1 gm. of powdered gelatin (going through mesh 30 but not through 60) were rendered isoelectric in the way described in previous publications. They were then put for 2 hours at 15°C. into 200 cc. of solutions of  $\text{LaCl}_3$  of different concentration, all of pH 4.7. In this time equilibrium was practically established between the gel and the outside  $\text{LaCl}_3$  solution. The suspension was then transferred into cylinders and the gelatin granules allowed to settle (at 15°C.). The supernatant liquid and the solid gelatin granules were then separated by filtration, the solid gelatin granules were melted by warming, poured into cylinders with two glass tubes attached (as described in

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 463.

a recent book<sup>2</sup>) and the gelatin was allowed to solidify at 15°C. The p.d. between the solid gelatin and the supernatant  $\text{LaCl}_3$  solution was then measured with two identical saturated KCl-calomel electrodes connected with a Compton electrometer as previously described.<sup>2</sup> Table I gives the observed p.d. The  $\text{LaCl}_3$  solutions used varied between  $M/24$  and  $M/98,304$ .

The gelatin was nearly but not entirely isoelectric, since it had a positive charge of about 15 millivolts. This means that its pH was not 4.7 but probably about 4.6 or possibly slightly less.

Table I shows that the p.d. rises upon the addition of increasing quantities of  $\text{LaCl}_3$  from 15 to 23.5 millivolts at a concentration of  $M/6,144$   $\text{LaCl}_3$ . When the concentration of  $\text{LaCl}_3$  rises beyond this point, the p.d. decreases again until it becomes 5.0 at a concentration of  $M/24$   $\text{LaCl}_3$ .  $\text{LaCl}_3$  acts, therefore, upon the p.d. of isoelectric gelatin in a similar way to HCl. In the case of HCl it has been proven that a salt is formed between gelatin (or any other isoelectric protein) in which the H ion becomes part of a complex positively charged protein ion while the Cl is the anion. It is natural to assume that the reaction is similar to that between  $\text{NH}_3$  and HCl where the salt  $\text{NH}_4\text{Cl}$  is formed, inasmuch as the protein contains  $\text{NH}_2$  or  $\text{NH}$  groups in which the N is still able to attract and hold electrostatically another H ion. The correctness of this view is supported by the fact that when we add HCl to a 1 per cent solution of gelatin and measure the Cl potential of the solution, the Cl potential is the same as when we add the same amount of HCl to the same quantity of water (without gelatin), while the H potential is considerably lower in the gelatin solution than in the pure aqueous solution.

When we add increasing concentrations of HCl to solid isoelectric gelatin, gelatin chloride is formed and the ionization of the protein leads to the establishment of a Donnan equilibrium between the solid gelatin and the surrounding aqueous solution, and the unequal distribution of the oppositely charged ions inside and outside the gel is responsible for the p.d.<sup>3</sup> The theory of membrane equilibria

<sup>2</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 154.

<sup>3</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 120 ff; *J. Gen. Physiol.*, 1920-21, iii, 667; 1921-22, iv, 351, 463.

demands that the p.D. increase with the increase in the concentration of gelatin ions formed, *i.e.* with the increase in the concentration of the acid added, and that the p.D. diminish with the increase of the concentration of Cl ions. At first, the augmenting influence of the acid (through formation of gelatin chloride) increases more rapidly with increasing concentration of acid than the depressing effect of the Cl ions, until a certain part of the gelatin is transformed into ions. From that point on the increase in p.D. due to the ionization of the gelatin increases less rapidly than the depressing effect of the Cl ions and the p.D. diminishes again when more acid is added. This has all been discussed more fully and need not be repeated here.<sup>3</sup>

The experiments mentioned in Table I show that  $\text{LaCl}_3$  acts in a way entirely similar to HCl. When  $\text{LaCl}_3$  solutions of low concentration (and of pH 4.7) are added to isoelectric gelatin, the p.D. rises with increasing concentration of  $\text{LaCl}_3$  until the concentration is M/6,144, when the p.D. falls again. This suggests that the La ion of the  $\text{LaCl}_3$  solution enters into combination with gelatin, as does the H in the case of HCl, by giving rise to gelatin-lanthanum chloride, in which the cation is a complex positively charged gelatin-La ion, while the anion is the Cl ion. The increase in ionization of the gelatin causes an increase in the p.D., while the Cl ion depresses the p.D. just as if HCl had been added. There is this difference between the gelatin chloride formed by the reaction between isoelectric gelatin and HCl and the gelatin chloride formed by the reaction between gelatin and  $\text{LaCl}_3$ ; namely, that the  $\text{LaCl}_3$  can be easily washed away while the HCl is held more tenaciously.<sup>4</sup> This is easily understood, since the radius of the La ion is so large that in spite of the high charge of the ion it is held with a considerably smaller force by the gelatin than is the H ion, which has no shell of electrons and whose positive nucleus can approach the N of the gelatin very closely.

The action of solutions of  $\text{Na}_4\text{Fe}(\text{CN})_6$  of pH 4.7 on isoelectric gelatin is similar to that of NaOH. The method was the same as that described in the case of  $\text{LaCl}_3$ . The results are given in Table II, showing that  $\text{Na}_4\text{Fe}(\text{CN})_6$  charges the gelatin negatively, the charge

<sup>4</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 27; *J. Gen. Physiol.*, 1918-19, i, 237.

TABLE I.  
P. D. between Solid Isoelectric Gelatin and Solutions of  $\text{LaCl}_3$  at pH near (but Probably Slightly Below) 4.7.  
Solid Gelatin Positively Charged.

Concentration of $\text{LaCl}_3$	0	M/98,304	M/49,152	M/24,576	M/12,288	M/6,144	M/3,072	M/1,536	M/768	M/384	M/192	M/96	M/48	M/24
P. D., millivolts ...	15.0	16.0	20.0	20.0	21.0	23.5	22.5	22.0	17.5	14.0	10.0	9.0	6.0	5.0

TABLE II.  
P. D. between Solid Isoelectric Gelatin and  $\text{Na}_4\text{Fe}(\text{CN})_6$  at pH near 4.7. Solid Gelatin Is Negatively Charged Where Minus Sign Is Added.

Concentration of $\text{Na}_4\text{Fe}(\text{CN})_6$	0	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
P. D., millivolts .....	15.0	-3.0	-9.0	-15.0	-22.0	-13.0	-13.0	-11.0	-8.5	-5.5	-4.0	-2.5	-1.5	-1.0

TABLE III.  
Influence of Different Concentrations of  $\text{NaCl}$ ,  $\text{Na}_2\text{SO}_4$ , and  $\text{CaCl}_2$  on the P. D. between Solid Gelatin and Outside Solution.  
Gelatin Always Positively Charged unless Minus Sign Is Added.

Concentration.	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1M
		millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts	millivolts
$\text{NaCl}$ .....	13.0		12.0	9.0	7.0	4.0	2.0	1.0	0	0	0	0	0	0.5	1.0
$\text{CaCl}_2$ .....			13.0	6.0	9.0	2.5	3.5	3.0	2.0	1.5	1.5	3.0	2.5	2.5	1.0
$\text{Na}_2\text{SO}_4$ .....			7.0	4.5	0.5	-2.0	-2.0	-2.0	-1.5						



increasing with an increase in the concentration of  $\text{Na}_4\text{Fe}(\text{CN})_6$  until the concentration of  $M/4,096$  is reached where the p.d. between solid gelatin and  $\text{Na}_4\text{Fe}(\text{CN})_6$  solution is 22 millivolts, the gelatin having the negative charge. Upon increasing the concentration of  $\text{Na}_4\text{Fe}(\text{CN})_6$  beyond  $M/4,096$  the p.d. diminished again until it became 1 millivolt when the concentration of  $\text{Na}_4\text{Fe}(\text{CN})_6$  was  $M/8$  (Table II).

The next step was to find whether solutions of  $\text{CaCl}_2$  of pH 4.7 also give a positive charge to isoelectric gelatin and whether solutions of  $\text{Na}_2\text{SO}_4$  give a negative charge. Table III shows that both of these salts, as well as  $\text{NaCl}$ , have only a depressing effect on the potential of isoelectric gelatin but cause no increase of p.d. in low concentration.

There is, however, a slight difference between the action of the three salts. While in the case of  $\text{Na}_2\text{SO}_4$  gelatin becomes slightly negative in concentrations beyond  $M/512$ , this does not happen in the case of  $\text{NaCl}$  and  $\text{CaCl}_2$ . The negative charge is, however, so small that it would not be wise to attribute much importance to this fact. The important fact is that solutions of  $\text{LaCl}_3$  and  $\text{Na}_4\text{Fe}(\text{CN})_6$  of pH 4.7 have a similar influence on isoelectric gelatin to that of acids and alkalis respectively, while this effect cannot be demonstrated in the case of  $\text{NaCl}$ ,  $\text{CaCl}_2$ , or  $\text{Na}_2\text{SO}_4$ . This would be intelligible on the basis of the assumption that the electrostatic attraction of  $\text{Na}$ ,  $\text{Cl}$ ,  $\text{SO}_4$ , and  $\text{Ca}$  ions is not sufficient to cause the formation of any considerable quantity of ionizable gelatin salts when reacting with isoelectric gelatin; at least not in the concentrations of salts used in this experiment. If this surmise is correct, we must consider the fact that the electrostatic attraction of an ion is a function of at least two variables; namely, the number of charges and the radius. If this is taken into consideration it is not to be expected that all divalent ions should be as inactive as  $\text{Ca}$  or  $\text{SO}_4$ .

## II.

If the influence of  $\text{LaCl}_3$  and  $\text{Na}_4\text{Fe}(\text{CN})_6$  on the p.d. of solid isoelectric gelatin is due to ionization of the protein and the establishment of a Donnan equilibrium as a consequence of this ionization, this should betray itself also in the osmotic pressure of mixtures of

isoelectric gelatin in solutions of  $\text{LaCl}_3$  or  $\text{Na}_4\text{Fe}(\text{CN})_6$  of pH 4.7. The osmotic pressure of 1 gm. of isoelectric gelatin in 100 cc. of solution should at first increase with the concentration of  $\text{LaCl}_3$  or  $\text{Na}_4\text{Fe}(\text{CN})_6$  until a maximum is reached, and the osmotic pressure should then drop again if the concentration of these two salts is increased beyond this point. On the other hand,  $\text{NaCl}$ ,  $\text{LiCl}$ ,  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ , or  $\text{Na}_2\text{SO}_4$  should have either no such effect, or it should be considerably smaller.

A stock solution of concentrated isoelectric gelatin was prepared and so diluted that 1 gm. (by dry weight) of such isoelectric gelatin was contained in 100 cc. of  $\text{H}_2\text{O}$  or various concentrations of the salts mentioned, all of a pH of 4.7. Collodion bags of a volume of about 50 cc. were filled with these gelatin solutions. Each bag was put into a beaker containing 350 cc. of a solution of the same salt and the same concentration as that in which the gelatin was dissolved. These 350 cc. of outside solutions were free from gelatin but had the same pH as the salt solution inside the bag; namely, 4.7. The osmotic pressure was measured in the way previously described and the final measurements were made after 18 or 20 hours. The temperature was kept constant, in some cases at  $24^\circ$ , but generally at  $27^\circ\text{C}$ . The higher temperature was chosen to prevent the gelatin from setting to a gel too quickly; *i.e.*, before osmotic equilibrium between the gelatin solution and the outside solution was established. Isoelectric gelatin is rather insoluble but becomes more soluble if salt is added. We shall discuss this more fully in a later part of this paper.

The results of these experiments are given in Table IV. From this table it is clear that the addition of  $\text{LaCl}_3$  and  $\text{Ce}(\text{NO}_3)_3$  or  $\text{Na}_4\text{Fe}(\text{CN})_6$  acts on the osmotic pressure of 1 per cent solutions of isoelectric gelatin in a similar way as the addition of acid or alkali, inasmuch as the addition of little  $\text{LaCl}_3$  or  $\text{Ce}(\text{NO}_3)_3$  or  $\text{Na}_4\text{Fe}(\text{CN})_6$  raises the osmotic pressure until a maximum is reached at a concentration of  $\text{M}/2,048$  for  $\text{LaCl}_3$  and of  $\text{M}/4,096$  for  $\text{Na}_4\text{Fe}(\text{CN})_6$ ; while the addition of more  $\text{LaCl}_3$  or  $\text{Ce}(\text{NO}_3)_3$  or  $\text{Na}_4\text{Fe}(\text{CN})_6$  depresses the osmotic pressure. None of the other salts used,  $\text{NaCl}$ ,  $\text{LiCl}$ ,  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ , or  $\text{Na}_2\text{SO}_4$ , acted in this way. There was possibly a slight rise when the concentration of these latter salts became very high.

These experiments confirm the conclusion reached in connection with the experiments on the charge of solid gelatin that salts with trivalent cations or tetravalent anions ionize isoelectric gelatin and thereby set up a Donnan equilibrium; while salts of the type of  $\text{CaCl}_2$  or  $\text{Na}_2\text{SO}_4$  or  $\text{NaCl}$  do not act this way.

In the experiments just described it seemed advisable to measure also the influence of different salt solutions of pH 4.7 on the p.d. between solutions of isoelectric gelatin inside the collodion bag and the outside aqueous solution free from gelatin but also of pH 4.7. These measurements were taken at the end of the experiments after

TABLE IV.

*Influence of Salt Solutions of pH 4.7 on the Osmotic Pressure (in Mm.  $\text{H}_2\text{O}$ ) of 1 Per Cent Solutions of Isoelectric Gelatin.*

Concentration.	0	M/65,536	M/32,768	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
$\text{Na}_4\text{Fe}(\text{CN})_6$ .....	18			51	94	95	90	75	63	51	48	41	43	38					
$\text{Na}_2\text{SO}_4$ .....	30						27	25	30	29	33	32	26	32	33	29			
$\text{NaCl}$ .....	23	22	22	23	23	22	23	27	28	31	25	29	26	28	28	30	35	38	
$\text{LiCl}$ .....							26	26	28	27	30	28	32	36	38	39	40	43	
$\text{MgCl}_2$ .....		23	20	24	27	23	28	24	28	28	27	29	33	31	33	36	40	43	42
$\text{CaCl}_2$ .....		22	25	24	26	28	26	30	32	29	27	32	30	31	33	40	38	38	40
$\text{LaCl}_3$ .....	27	26	32	40	51	52	60	62	57	57	54	50	48						
$\text{Ce}(\text{NO}_3)_3$ .....	23	28	34	46	50	58	71	69	63	55	50	45							

osmotic equilibrium was established. The figures show that the addition of little  $\text{LaCl}_3$  or  $\text{Ce}(\text{NO}_3)_3$  increases the p.d. across the membrane until a maximum is reached at a concentration near M/8,192 or M/4,096 and that the p.d. drops again with a further increase in the concentration of the salt. The gelatin is positively charged, showing that the gelatin forms cations.  $\text{Na}_4\text{Fe}(\text{CN})_6$  acts similarly except that gelatin is negatively charged.  $\text{NaCl}$ ,  $\text{LiCl}$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{MgCl}_2$ , and  $\text{CaCl}_2$  create no potential difference; or in other words, they cause no ionization of isoelectric gelatin. These results agree with the results obtained with solid gelatin and they agree also with the results obtained in the experiments on anomalous osmosis (Table V).

TABLE V.  
Influence of Salt Solutions of pH 4.7 on Membrane Potentials in Millivolts between Solutions of Isoelectric Gelatin and Aqueous Solutions Free from Gelatin. Gelatin Solution Positive unless Minus Sign Is Added.

Concentration.	M/65.536	M/32.768	M/16.384	M/8.192	M/4.096	M/2.048	M/1.024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
Na <sub>4</sub> Fe(CN) <sub>6</sub> .....	-3.0		-9.0	-13.5	-10.0	-6.0	-4.0	-3.0	-1.5	0	0	-0.5	-0.5					
Na <sub>2</sub> SO <sub>4</sub> .....	-1.0					-0.5	0	-0.5	0	0	0	0	0	0	0	0	0	
NaCl.....	1.0		3.0	2.0	0.5	1.0	0.5	0	0	-0.5	0	0	-0.5	0	0	0	0	
LiCl.....	0.5					0	-1.0	-0.5	0	0	0	0	-0.5	0	0	0	0	
MgCl <sub>2</sub> .....			0	0	0	0	0	0	0	0	0	0	0	-0.5	-0.5	-1.0	-1.5	0
CaCl <sub>2</sub> .....	-1.0		0.5	1.0	1.5	1.5	0.5	0.5	0.5	0	0	0	0	0	-0.5	-0.5	-0.5	-1.0
LaCl <sub>3</sub> .....	-0.5	1.0	5.0	8.0	5.0	4.0	3.5	3.0	2.0	1.5	1.0	1.0	0	0	-0.5	-0.5	-0.5	-1.0
Ce(NO <sub>3</sub> ) <sub>3</sub> .....	-2.5	1.0	5.0	6.5	7.5	7.0	5.0	3.5	2.0	1.0	0.5							

TABLE VI.  
Influence of Solutions of LaCl<sub>3</sub> on pH of 1 Per Cent Solutions of Nearly Isoelectric Gelatin.

Concentration of LaCl <sub>3</sub> .....	0	M/16,384	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64
pH.....	4.85	4.80	4.78	4.70	4.64	4.57	4.52	4.45	4.39	4.34

## III.

While all this seems clear, there remains the possibility that the effects of  $\text{LaCl}_3$  and of  $\text{Na}_4\text{Fe}(\text{CN})_6$  on the p.d. and osmotic pressure of isoelectric gelatin are due to a different kind of ionization from that assumed above. When a solution of  $\text{LaCl}_3$  of pH 4.7 is added to a gelatin solution of the same pH, the solution becomes more acid; and when a solution of  $\text{Na}_4\text{Fe}(\text{CN})_6$  of pH 4.7 is added to a solution of isoelectric gelatin, the solution becomes more alkaline. A solution of  $\text{LaCl}_3$  of pH 4.7 makes, therefore, isoelectric gelatin more acid and this should lead to a formation of gelatin chloride, without the necessity of assuming the formation of a complex gelatin-La cation; while solutions of  $\text{Na}_4\text{Fe}(\text{CN})_6$  of pH 4.7 make isoelectric gelatin more alkaline and this should lead to a formation of Na gelatinate. To test this possibility 1 per cent solutions of nearly isoelectric gelatin (pH 4.85) were made up in various concentrations of  $\text{LaCl}_3$  of pH 4.7 and the pH was determined electrometrically (Table VI).

According to Table V the highest p.d. is reached in  $\text{LaCl}_3$ -gelatin mixtures at a concentration of  $\text{LaCl}_3$  of  $\text{m}/8,192$ ; such a solution influences the pH of the gelatin solution too little to account for the p.d. of 8.0 millivolts, since near the isoelectric point the difference between pH inside and outside is almost zero.

It was then attempted to repeat the experiments on the influence of  $\text{Ce}(\text{NO}_3)_3$  on the p.d. and osmotic pressure of solutions of isoelectric gelatin in buffer solutions, but these experiments failed since the addition of the salt contained in the buffer solution in itself sufficed to suppress the p.d. to be expected.

To reach a decision the following experiments were made.

1 per cent solutions of isoelectric gelatin were prepared and to some solutions was added HCl to others NaOH so that the pH values obtained were approximately 3.5, 4.0, 4.4, 4.7, 5.0, and 5.5. These gelatin solutions were made up in solutions of different salts of the same pH. In this way it was possible to ascertain the influence of these salts on the pH of the gelatin solution (Table VII).

The p.d. between these gelatin solutions inside the collodion bags and the outside aqueous solutions of the same salt and originally

of the same pH as the gelatin solution (but without gelatin), was also measured after osmotic equilibrium was established. The figures for the p.D. are always under the figures for the pH of the gelatin-salt solutions. The result is very striking. The gelatin is always positively charged when the gelatin is dissolved in  $m/768$   $\text{LaCl}_3$  regardless of the pH. Even when the pH of the solution is on the alkaline side of the isoelectric point the gelatin is positively charged in the presence of  $m/768$   $\text{LaCl}_3$ . No such result is observed when the salt added is  $\text{NaCl}$ ,  $\text{CaCl}_2$ , or  $\text{Na}_2\text{SO}_4$ . In this case the p.D. across the membrane was zero at pH 4.7 and 4.8 and the gelatin became negative when the pH became 5.0 or above. This shows that the positive charge of isoelectric gelatin in the presence of

TABLE VII.

		Gelatin-acid salts.			Isoelec- tric gelatin.	Metal gelatinates.	
$m/768$ $\text{LaCl}_3$ .	pH.....	3.66	3.98	4.32	4.72	4.82	5.3
	p.D., millivolts.....	+8.5	+7.0	+5.5	+3.5	+3.0	+2.0
$m/512$ $\text{CaCl}_2$ .	pH.....	3.62	4.02	4.44	4.72	5.02	5.43
	p.D., millivolts.....	+8.5	+5.5	+2.5	0	-1.0	-2.5
$m/256$ $\text{NaCl}$ .	pH.....	3.66	3.98	4.49	4.80	5.19	5.68
	p.D., millivolts.....	+9.5	+7.0	+2.5	0	-3.0	-6.0
$m/512$ $\text{Na}_2\text{SO}_4$ .	pH.....	3.63	4.06	4.41	4.83	5.16	5.82
	p.D., millivolts.....	+6.0	+4.0	+1.0	0	-2.0	-3.0

$\text{LaCl}_3$  of pH 4.7 cannot be due to the change in pH but must be due to some other cause of ionization of gelatin such as the formation of a complex gelatin-La cation.

The pH for  $\text{Na}_4\text{Fe}(\text{CN})_6$  could not be measured electrometrically.

In view of all these results we must draw the conclusion that salts with trivalent (and probably also tetravalent) cations cause isoelectric gelatin and gelatin on the alkaline side of the isoelectric point (but near this point) to assume a positive charge owing to the formation of positively charged protein ions, probably of the type gelatin-La. This explains the reversal of the charge of gelatin by trivalent cations observed in anomalous osmosis and kindred phenomena. Tetravalent anions confer a negative charge on isoelectric gelatin and the mechanism is probably similar. Changes of

hydrogen ion concentration may enter to some extent into these effects but they are probably of only secondary importance. No such effects have thus far been obtained with salts possessing divalent or monovalent ions; *i.e.*, salts of the type  $\text{Na}_2\text{SO}_4$ ,  $\text{CaCl}_2$ , and  $\text{NaCl}$ .

#### IV.

In a gelatin chloride solution of pH 3.0, the greater part of the gelatin is ionized, and since the H ion is held more firmly by the gelatin than the La ion, the addition of  $\text{LaCl}_3$  to a solution of gelatin chloride of pH 3.0 should not have any augmenting effect on the p.d. or the osmotic pressure of the gelatin chloride solution. The only effect the addition of  $\text{LaCl}_3$  to a solution of gelatin chloride should have is the depressing effect of the Cl ions. Hence when we mix a solution of gelatin chloride of pH 3.0 with solutions of  $\text{LaCl}_3$  of the same pH the effect on the p.d. and the osmotic pressure should be merely a depression of the values of these latter properties, and the depression should be quantitatively identical with the effect of  $\text{CaCl}_2$  or  $\text{NaCl}$  solutions of the same concentration of Cl ions and the same pH.

This fact had already been ascertained in previous experiments already published,<sup>3</sup> but since these direct measurements of p.d. are of such importance for the theory of the origin of the p.d. between colloids and aqueous solutions it seemed advisable to repeat them. 1 gm. of gelatin chloride of pH 3.0 was dissolved in 100 cc. of solutions of various salts all brought to pH 3.0 through the addition of HCl. Collodion bags were filled with these solutions of gelatin chloride in different salts and each bag was dipped into 350 cc. of an aqueous solution of the same concentration of the same salt and the same pH as that inside the collodion bag, but without gelatin. Water diffused into the collodion bag until osmotic equilibrium was established, and the next day the final osmotic pressure and the p.d. between gelatin solution and outside aqueous solution were measured. The solutions of the three salts were prepared in such a way as to have the same concentration of Cl. In Fig. 1 are plotted the p.d. as ordinates over the concentration of the Cl ions as abscissæ. It is obvious that the influence of  $\text{NaCl}$ ,  $\text{CaCl}_2$ , and  $\text{LaCl}_3$

on the p.d. is identical for the same concentration of Cl. This proves that only the Cl ion of these salts influences the p.d. of a gelatin chloride solution of pH 3.0 and that the La ion does not increase the p.d. of the solution.

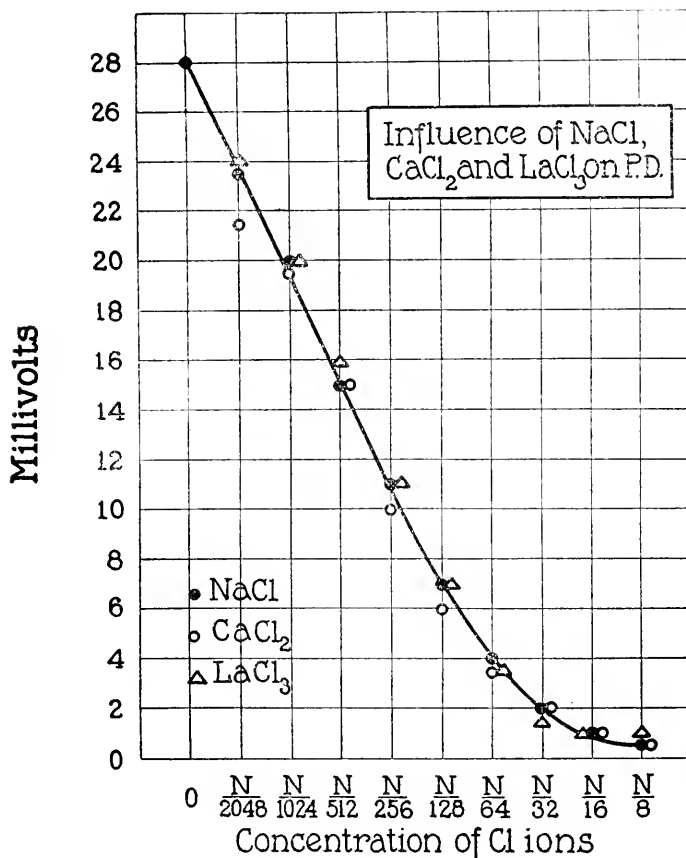


FIG. 1. Depressing influence of NaCl, CaCl<sub>2</sub>, and LaCl<sub>3</sub> on the p.d. between a 1 per cent solution of gelatin chloride of pH about 3.0 and aqueous solutions of the salts originally of the same pH, the two solutions being separated by colloidal membranes. Ordinates are the p.d., abscissæ the concentrations of Cl ions of the salts. The depressing effect of the three salts is the same for the same concentration of Cl ions, proving that the cations do not influence the p.d. of gelatin chloride solutions.



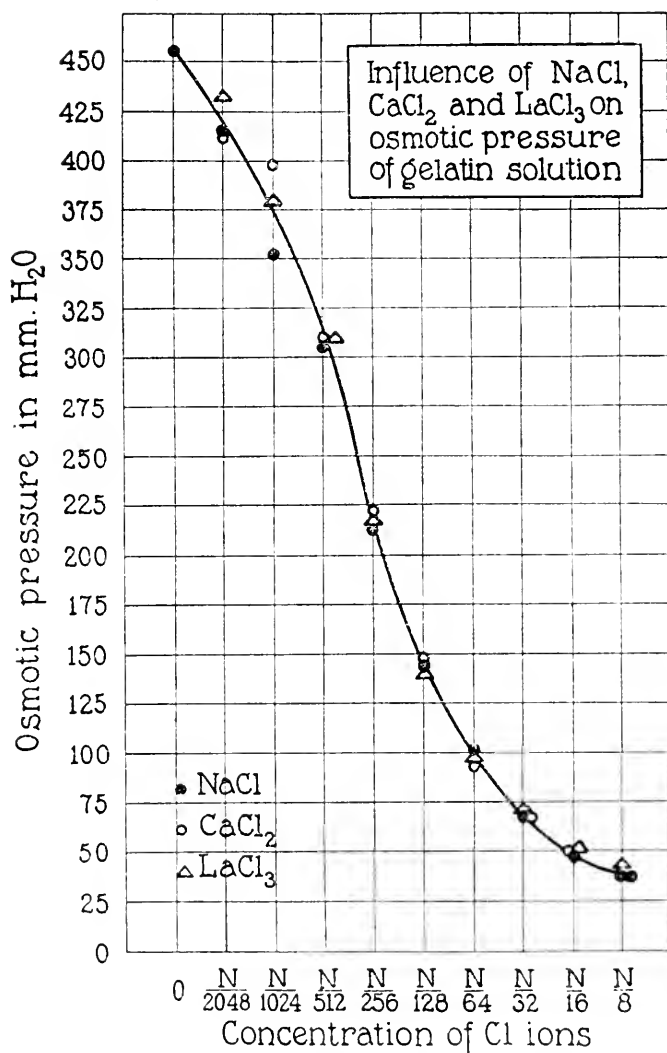


FIG. 2. Influence of NaCl, CaCl<sub>2</sub>, and LaCl<sub>3</sub> on osmotic pressure of a 1 per cent solution of gelatin chloride of pH 3.0. Ordinates are osmotic pressure in mm. H<sub>2</sub>O, abscissæ the concentrations of Cl ions of the salts. The depressing effect is the same for the three salts, proving that only the anion influences the osmotic pressure in this case.

TABLE VIII.  
*Influence of NaCl on the p.d. of 1 Per Cent Solutions of Gelatin Chloride, pH 3.0.*

Concentration of NaCl.	0	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
pH inside.....	3.35	3.29	3.25	3.21	3.15	3.09	3.06	3.05	3.05	3.06
pH outside.....	2.88	2.89	2.90	2.93	2.95	2.96	2.98	3.01	3.02	3.05
pH inside minus pH outside.....	0.47	0.40	0.35	0.28	0.20	0.13	0.08	0.04	0.03	0.01
p.d. calculated, millivolts.....	+28.0	+23.5	+20.5	+16.5	+12.0	+7.5	+4.5	+2.5	+1.8	+0.6
p.d. observed, millivolts.....	+28.0	+23.5	+20.0	+15.0	+11.0	+7.0	+4.0	+2.0	+1.0	+0.5

TABLE IX.  
*Influence of CaCl<sub>2</sub> on the p.d. of 1 Per Cent Solutions of Gelatin Chloride, pH 3.0.*

Concentration of CaCl <sub>2</sub> .	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16
pH inside.....	3.34	3.30	3.24	3.20	3.13	3.09	3.06	3.04	3.04	3.03
pH outside.....	2.88	2.90	2.91	2.94	2.93	2.96	2.99	2.99	3.01	3.01
pH inside minus pH outside.....	0.46	0.40	0.33	0.26	0.20	0.13	0.07	0.05	0.03	0.02
p.d. calculated, millivolts.....	+27.0	+23.5	+19.5	+15.5	+11.5	+7.5	+4.0	+3.0	+1.8	+1.2
p.d. observed, millivolts.....	+28.0	+21.5	+19.5	+15.0	+10.0	+6.0	+3.5	+2.0	+1.0	+0.5



In these experiments the hydrogen ion concentrations of the inside and outside solutions were measured and the writer begs leave to give these results (Tables VIII, IX, and X), since they prove once more that the P.D. between a solution of gelatin chloride and an outside solution across a collodion membrane is determined by the difference in the pH in the inside and outside solutions, as the Donnan theory demands. In these experiments the influence of the three salts on the osmotic pressure of the gelatin chloride solutions was also measured and the results are given in Fig. 2 showing that the osmotic pressure of a gelatin chloride solution of pH 3.0 is influenced only by the anion but not by the cation of the salt, since the effect of the three salts on the osmotic pressure of the solution is exactly the same when plotted over the concentration of the Cl ions. At this pH, therefore, the La does not increase the osmotic pressure of gelatin chloride solutions.

These observations support the idea that trivalent and tetravalent ions are able to transfer their charge to isoelectric protein by causing the protein to be ionized; probably in such a way that the trivalent or tetravalent ion is part of a complex protein ion.

#### SUMMARY AND CONCLUSIONS.

1. Experiments on anomalous osmosis suggested that salts with trivalent cations, *e.g.*  $\text{LaCl}_3$ , caused isoelectric gelatin to be positively charged, and salts with tetravalent anions, *e.g.*  $\text{Na}_4\text{Fe}(\text{CN})_6$ , caused isoelectric gelatin to be negatively charged. In this paper direct measurements of the P.D. between gels of isoelectric gelatin and an aqueous solution as well as between solutions of isoelectric gelatin in a collodion bag and an aqueous solution are published which show that this suggestion was correct.

2. Experiments on anomalous osmosis suggested that salts like  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ ,  $\text{NaCl}$ ,  $\text{LiCl}$ , or  $\text{Na}_2\text{SO}_4$  produce no charge on isoelectric gelatin and it is shown in this paper that direct measurements of the P.D. support this suggestion.

3. The question arose as to the nature of the mechanism by which trivalent and tetravalent ions cause the charge of isoelectric proteins. It is shown that salts with such ions act on isoelectric gelatin in a way

similar to that in which acids or alkalies act, inasmuch as in low concentrations the positive charge of isoelectric gelatin increases with the concentration of the  $\text{LaCl}_3$  solution until a maximum is reached at a concentration of  $\text{LaCl}_3$  of about  $\text{M}/8,000$ ; from then on a further increase in the concentration of  $\text{LaCl}_3$  diminishes the charge again. It is shown that the same is true for the action of  $\text{Na}_4\text{Fe}(\text{CN})_6$ . From this it is inferred that the charge of the isoelectric gelatin under the influence of  $\text{LaCl}_3$  and  $\text{Na}_4\text{Fe}(\text{CN})_6$  at the isoelectric point is due to an ionization of the isoelectric protein by the trivalent or tetravalent ions.

4. This ionization might be due to a change of the pH of the solution, but experiments are reported which show that in addition to this influence on pH,  $\text{LaCl}_3$  causes an ionization of the protein in some other way, possibly by the formation of a complex cation, gelatin- $\text{La}$ .  $\text{Na}_4\text{Fe}(\text{CN})_6$  might probably cause the formation of a complex anion of the type gelatin- $\text{Fe}(\text{CN})_6$ . Isoelectric gelatin seems not to form such compounds with  $\text{Ca}$ ,  $\text{Na}$ ,  $\text{Cl}$ , or  $\text{SO}_4$ .

5. Solutions of  $\text{LaCl}_3$  and  $\text{Na}_4\text{Fe}(\text{CN})_6$  influence the osmotic pressure of solutions of isoelectric gelatin in a similar way as they influence the p.D., inasmuch as in lower concentrations they raise the osmotic pressure of the gelatin solution until a maximum is reached at a concentration of about  $\text{M}/2,048$   $\text{LaCl}_3$  and  $\text{M}/4,096$   $\text{Na}_4\text{Fe}(\text{CN})_6$ . A further increase of the concentration of the salt depresses the osmotic pressure again.  $\text{NaCl}$ ,  $\text{LiCl}$ ,  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ , and  $\text{Na}_2\text{SO}_4$  do not act in this way.

6. Solutions of  $\text{LaCl}_3$  have only a depressing effect on the p.D. and osmotic pressure of gelatin chloride solutions of pH 3.0 and this depressing effect is quantitatively identical with that of solutions of  $\text{CaCl}_2$  and  $\text{NaCl}$  of the same concentration of  $\text{Cl}$ .



# IONIZING INFLUENCE OF SALTS WITH TRIVALENT AND TETRAVALENT IONS ON CRYSTALLINE EGG ALBUMIN AT THE ISOELECTRIC POINT.

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## I.

### INTRODUCTION.

Measurements of the membrane potentials between aqueous protein solutions or gels and surrounding water at equilibrium have yielded the result that salts with trivalent cations give isoelectric protein a positive charge while salts with tetravalent anions give it a negative charge.<sup>1</sup> On the basis of Donnan's theory of membrane potentials it was assumed that salts with trivalent cations, *e.g.*  $\text{LaCl}_3$ , form with isoelectric protein ionizable salts which result in the formation of positive protein-La ions and negative Cl ions; and that salts like  $\text{Na}_4\text{Fe}(\text{CN})_6$  form with isoelectric protein salts which result in the formation of negative protein-Fe  $(\text{CN})_6$  ions and positive Na ions.<sup>2</sup> In other words, salts with trivalent cations react with isoelectric protein like acids, and salts with tetravalent anions react with isoelectric protein like alkalies; with this difference, however, that the compounds of isoelectric gelatin with acids and bases are much more stable than those with the salts of trivalent cations or tetravalent anions. Salts with divalent ions like  $\text{Na}_2\text{SO}_4$ ,  $\text{CaCl}_2$ , or salts with monovalent ions like NaCl, did not produce any measurable charge on isoelectric gelatin in aqueous solutions. Experiments on anomalous osmosis through gelatin-collodion membranes were in harmony with these results.<sup>3</sup>

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 741.

<sup>2</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 165.

<sup>3</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 463.

It seemed of interest to find out whether experiments on the stability of aqueous solutions and suspensions of proteins at the isoelectric point are also in harmony with the results of the direct measurements of the membrane potentials. The reason why experiments on stability and flocculation were selected was that it is often stated that the flocculation of colloids is influenced in an opposite sense by the two oppositely charged ions of a salt, the ion with the same sign of charge as the colloid increasing the stability, the salt ion with the opposite sign of charge to that of the colloidal particle diminishing the stability of the suspension.

If we wish to use observations on the influence of salts on the stability of protein solutions at the isoelectric point for conclusions concerning the influence of ions on the electrical charges of particles, we are confronted with the difficulty that the electrical charges of particles are not the only forces which keep proteins in solution. There are two different kinds of forces determining the stability of solutions or suspensions of proteins, namely; first, the attraction between the molecules of the protein and the solvent, and second, forces of electrostatic repulsion between micellæ. When the forces of attraction between molecules of the solvent and molecules of the solute (which may be secondary valency forces) are greater than the forces of attraction between the molecules of the solute for each other, the solution will be stable. This type of forces acts in the general case of solutions of crystalloids.

When the forces of attraction between the molecules of solute and solvent are weak, the molecules of the solute upon colliding may adhere to each other and aggregates will be formed. This aggregate formation will lead to a flocculation or coagulation of the whole mass unless new forces originate in the small nascent aggregates (or micellæ) which prevent their coalescence into larger aggregates. These forces may be electrical charges whereby the nascent micellæ repel each other. The writer has investigated the origin of these charges in the case of protein micellæ and has found that they are due to the establishment of a Donnan equilibrium between particles and solution.<sup>4</sup> A membrane equilibrium between particles and

<sup>4</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 120; *J. Gen. Physiol.*, 1920-21, iii, 667; 1921-22, iv, 351.



solution can, however, only be established when the particles contain protein ions.

There exists a criterion which seems to permit us to decide which one of the two types of forces is responsible for the stability of a solution or suspension. When the stability depends upon the repulsive forces due to a potential difference between micellæ and solution, comparatively low concentrations of solutions will be required for the precipitation of the protein, since comparatively low concentrations of salts (*e.g.* concentrations of  $M/8$  or less) suffice for the annihilation of the P.D. When, however, the stability of a solution is not determined by the P.D. between micellæ and solution but by forces of residual valency between molecules of solute and solvent, much higher concentrations of salts are, as a rule, required for precipitation than are sufficient for the annihilation of a P.D. caused by the Donnan equilibrium. Moreover, the efficiency of a salt in annihilating the P.D. between a micella and a solution is the lower the higher the valency of that ion of the salt which has the opposite sign of charge to that of the micella.

## II.

### *The Prevention of Heat Coagulation of Isoelectric Egg Albumin by Trivalent and Tetravalent Ions.*

Isoelectric crystalline egg albumin is quite soluble in water as long as the temperature is low. 8 per cent solutions kept at 2°C. remained perfectly clear for more than a year—and they would probably have kept clear indefinitely. Since it requires very high concentrations of salts to cause a precipitation of isoelectric crystalline egg albumin from aqueous solution at ordinary temperature, we may assume that the forces determining the stability of solutions of isoelectric crystalline egg albumin at sufficiently low temperature are not the electrical charges of micellæ but the attraction between molecules of isoelectric albumin and molecules of water. When, however, the temperature of a 1 per cent solution of isoelectric crystalline egg albumin is raised to about 73°C. or above, crystalline egg albumin is flocculated. Through the rise in temperature a change occurs in the molecule of crystalline egg albumin, whereby the attraction of the molecules of

albumin for each other becomes greater than the attraction between the molecules of albumin and water.

If the albumin is practically non-ionized (as is the case at the isoelectric point) no Donnan equilibrium between the nascent micellæ and the surrounding solution can be established and no p.d. between the nascent micellæ and the solution can prevent the coalescence of the micellæ. When, however, part of the albumin is ionized, the molecules of albumin will also unite upon heating to form micellæ, but these micellæ will begin to repel each other as soon as they contain protein ions. For in this case the protein ions in the nascent micellæ will cause the establishment of a Donnan equilibrium between the micellæ and the solution, and the electrical charge produced thereby on the particles will prevent the further coalescence of the nascent micellæ. This charge will increase with the relative concentration of ionized protein contained in the micellæ. It is obvious that the average size of the micellæ will remain the smaller the greater the relative concentration of protein ions in solution; since the greater the relative concentration of ionized protein the smaller will be the average number of protein molecules which can form an aggregate without including protein ions. This argument is supported by the well known fact that when we add some acid or alkali to isoelectric albumin, the solution will become only opalescent on heating but heat precipitation of the albumin will no longer occur. A comparison of the effect of increasing concentrations of acid shows that the relative size of the micellæ will become the smaller the greater the relative mass of ionized protein. To demonstrate this, 10 cc. of an aqueous 0.2 per cent solution of almost isoelectric crystalline egg albumin and containing varying amounts of 0.1 *N* HCl were put into test-tubes, and these test-tubes were put into boiling water until the temperature of the albumin solution rose to 90°C. Then the test-tubes were allowed to cool at room temperature and the appearance of the solution was noticed. Table I gives the result.

When the 10 cc. contained 0.01 cc. of 0.1 *N* HCl the protein remained practically isoelectric (pH 4.8), practically no ionization was produced, and hence flocculation occurred upon heating.



The addition of 0.02 cc. of 0.1 *N* HCl prevented coagulation but the solution was opaque showing that only when the micellæ were comparatively large did they assume electrical charges; owing to the fact that the concentration of albumin ions was small compared with that of non-ionized protein. These charges sufficed, however, to prevent the further coalescence of the large micellæ. When the solutions contained 0.03 cc. of 0.1 *N* HCl the relative concentration of ionized protein was increased and hence the micellæ remained smaller; the solution was no longer opaque but opalescent. With the addition of 0.04 cc. of 0.1 *N* HCl the solution became very transparent, showing only slight opalescence. With the increasing concentration of ionized protein the average number of molecules in a micella was considerably diminished, and this small size of the average micellæ manifested itself in the greater transparency of the solution. With a still greater concentration of HCl the average size of the micellæ diminished still further and the solution became as clear as water.

When, however, the concentration of HCl was increased beyond a certain limit, the p.d. between the micellæ and solution was diminished again on account of the depressing effect of the Cl ions demanded by Donnan's theory. When 100 cc. of 1 per cent solution of originally isoelectric albumin contained 30 cc. of *N* HCl, the protein coagulated at a temperature of 66°C. In this case all the protein was practically ionized but the p.d. between the micellæ and the liquid was nevertheless depressed to zero on account of the high concentration of Cl ions.

By measuring the concentration of salt required to precipitate crystalline egg albumin from a 1 per cent solution in water of pH 3.0 at a temperature of 70°C. we can show that the forces preventing heat coagulation in this case are the electrical charges of the micellæ, since the concentration of salt required to cause precipitation is of the order of  $M/8$  or below, and since sulfates are more efficient than chlorides.

The fact that ionization of protein prevents heat coagulation of albumin can be used to find out whether other electrolytes than acids or alkalis are able to produce ionization of isoelectric egg

albumin. If other ions, like La, Ca, Na,  $\text{SO}_4$ , have such an effect on aqueous solutions of isoelectric albumin, it should show itself in the prevention of heat coagulation and in the optical appearance of the albumin solution after heating.

The experimental procedure was as follows: 7 cc. of water of pH 4.8 (this pH being the isoelectric point of crystalline egg albumin) were added to 2 cc. of 1 per cent solution of isoelectric crystalline egg albumin (of course, also of pH 4.8) and then 1 cc. of a salt solution containing different salts of different concentration, but always of pH 4.8, was added. The test-tubes containing the 10 cc. of the mixtures were put into boiling water until the liquid in the test-tubes reached a temperature of  $90^\circ\text{C}$ . and then the test-tubes were taken out of the water bath and allowed to cool at room temperature. Table II gives the appearance of the various mixtures after standing over night.

These experiments show first that the heat coagulation of isoelectric solutions of crystalline egg albumin is prevented by the addition of low concentrations of  $\text{LaCl}_3$  or  $\text{Na}_4\text{Fe}(\text{CN})_6$  of pH 4.8. The concentration of  $\text{LaCl}_3$  sufficient for this purpose was  $\text{M}/5,000$  and that of  $\text{Na}_4\text{Fe}(\text{CN})_6$  about the same. Hence these two salts acted on the heat coagulation of isoelectric egg albumin like acids or alkali respectively. Moreover, it is obvious from Table II that at first the size of the micellæ formed diminishes with increasing concentration of  $\text{LaCl}_3$  ions until the molecular concentration of  $\text{LaCl}_3$  is about  $\text{M}/160$ . With a further increase of concentration of salt the size of the micellæ increases again (at  $3\text{ M}/80$ ) owing to the fact that the p.d. is depressed by the Cl ions; and at  $\text{M}/20$   $\text{LaCl}_3$  this depressing action of the Cl ions on the p.d. is sufficient to permit again the heat coagulation of the albumin. In the case of  $\text{Na}_4\text{Fe}(\text{CN})_6$  the solution ceases to be clear when the concentration becomes  $6\text{ M}/80$ ; in this case the depressing action of the Na ions on the p.d. of the negatively charged micellæ is so great that the micellæ begin to coalesce again.

None of the other salts tried,  $\text{CaCl}_2$ ,  $\text{BaCl}_2$ ,  $\text{NaCl}$ , or  $\text{Na}_2\text{SO}_4$ , is able to prevent heat coagulation of isoelectric egg albumin in an aqueous solution of pH 4.8. It is, of course, possible that certain

TABLE II.  
*Influence of Different Salts on Heat Coagulation of Crystalline Egg Albumin in Aqueous Solution at pH of Isoelectric Point.*

Total concentration of salt in 10 cc. of 0.2 per cent albu- min.....	10 M/80	8 M/80	6 M/80	4 M/80	3 M/80	2 M/80	M/80	M/160	M/320	M/640	M/1,280	M/2,560	M/5,120	M/10,240	M/20,480	M/40,960	M/81,920	0	
LaCl <sub>3</sub> .....	Coagulated.				Opales- cent.	Bluish clear. Clearest.							Opales- cent and turbid.	Milky.	Coagulated.				
Na <sub>4</sub> Fe (CN) <sub>6</sub> ....	Very opaque.	Slight opales- cence.	Clear.			Increasing opalescence. ↑							Milky.	Co- agu- lated.					
BaCl <sub>2</sub> .....	Coagulated.					Coagulated.													
CaCl <sub>2</sub> .....	Coagulated.					Coagulated.													
Na <sub>2</sub> SO <sub>4</sub> .....	Coagulated.					Coagulated.													
NaCl.....	Coagulated.					Coagulated.													

other bivalent and monovalent ions act differently, since the valency is not the only variable determining the combining action.<sup>5</sup>

These results are in entire agreement with the experiments published in a preceding paper<sup>1</sup> showing that only salts with trivalent and tetravalent ions can produce a membrane potential on isoelectric gelatin while Na, Ca, Ba, and SO<sub>4</sub> have no such effect.

When crystalline egg albumin is dissolved in a solution with little water and much alcohol, salts have not the same influence on the stability of the solution that they have in an aqueous solution. This is due to the difference in the nature of the solvent, since the influence of salts on the stability of such alcoholic solutions of crystalline egg albumin is similar to the influence of salts on the stability of solutions of gelatin in a solution with much alcohol and little water. The stability of isoelectric gelatin in a mixture with little water and much alcohol is increased not only by salts with trivalent and tetravalent ions but also by salts with bivalent ions, such as MgCl<sub>2</sub>, CaCl<sub>2</sub>, SrCl<sub>2</sub>, BaCl<sub>2</sub>, and Na<sub>2</sub>SO<sub>4</sub>, while salts like MgSO<sub>4</sub>, LiCl, NaCl, or KCl have no such effect. The clearing effect of Ba was considerably greater than that of Mg. We know too little about the p.d. and solubility in alcoholic solutions and for this reason the publication and discussion of these results may be postponed.

#### SUMMARY AND CONCLUSION.

1. While crystalline egg albumin is highly soluble in water at low temperature at the pH of its isoelectric point, it is coagulated by heating. It has long been known that this coagulation can be prevented by adding either acid or alkali, whereby the protein is ionized.

2. It is shown in this paper that salts with trivalent or tetravalent ions, *e.g.* LaCl<sub>3</sub> or Na<sub>4</sub>Fe(CN)<sub>6</sub>, are also able to prevent the heat coagulation of albumin at the isoelectric point (*i.e.* pH 4.8), while salts with a divalent ion, *e.g.* CaCl<sub>2</sub>, BaCl<sub>2</sub>, Na<sub>2</sub>SO<sub>4</sub>, or salts like NaCl, have no such effect.

<sup>5</sup> Michaelis, L., and Rona, P., *Biochem. Z.*, 1919, xciv, 225.

3. This is in harmony with the fact shown in a preceding paper that salts with trivalent or tetravalent ions can cause the ionization of proteins at its isoelectric point and thus give rise to a membrane potential between micellæ of isoelectric protein and surrounding aqueous solution, while the above mentioned salts with divalent and monovalent ions have apparently no such effect.



# ON THE INFLUENCE OF AGGREGATES ON THE MEMBRANE POTENTIALS AND THE OSMOTIC PRESSURE OF PROTEIN SOLUTIONS.

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## I.

When a protein chloride solution is separated by a collodion membrane from a dilute HCl solution free from protein, under suitable experimental conditions an osmotic equilibrium is reached after about 6 hours. When at that time the hydrogen ion concentrations of the protein solution and the outside solution are measured, it is found that the hydrogen ion concentration of the outside solution is higher than that of the protein solution, while the chlorine ion concentration is higher in the protein solution than outside.<sup>1</sup> This unequal concentration of the oppositely charged H and Cl ions inside and outside leads to a membrane potential and modifies the osmotic pressure of the protein solution. The values of both effects can be calculated from the differences in the pH (or pCl) inside and outside on the basis of Donnan's equation for membrane equilibria. The agreement between observed and calculated values on the basis of Donnan's equilibrium equation is excellent for the membrane potentials and equally good for the osmotic pressure, except that a slight difference in pH (in the second decimal) has a much greater influence on the calculated values of osmotic pressure than of the P.D. These facts were based on the writer's observations on solutions of gelatin and crystalline egg albumin<sup>1</sup> and they were confirmed by Hitchcock's observations on solutions of edestin.<sup>2</sup>

The writer has shown that if in a solution of gelatin chloride of a certain pH, part of the gelatin in solution is replaced by the

<sup>1</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922; *J. Gen. Physiol.*, 1920-21, iii, 667, 691.

<sup>2</sup> Hitchcock, D. I., *J. Gen. Physiol.*, 1921-22, iv, 597.

same weight of powdered gelatin (without changing the pH) the osmotic pressure of the solution is lowered, and this lowering of the osmotic pressure increases the more the more of the dissolved gelatin is replaced by powdered gelatin.<sup>3</sup> This shows that only that part of the protein which is in true solution, *i.e.* which does not exist in the form of larger aggregates, influences the osmotic pressure of a solution. The question arose whether or not powdered particles of gelatin would influence the membrane potential. To obtain an answer to this question the following experiment was made.

Powdered gelatin going through the meshes of sieve 30 but not through 60 was rendered isoelectric in the way previously described. Part of this isoelectric gelatin was melted and the melted and powdered isoelectric gelatin were mixed. The total weight of isoelectric gelatin in 100 cc. solution was always the same, but the proportion of powdered to dissolved gelatin varied as indicated in Table I. Thus when the weight of the powdered gelatin was 0.5 gm., the weight of the dissolved gelatin was about 0.3 gm.; when the weight of the powdered gelatin was 0.2 gm., that of the dissolved was 0.6 gm., etc. 100 cc. of the mixture contained 8 cc. of 0.1 N HCl, and the pH of the gelatin solution (at the equilibrium condition to be described) was between 3.2 and 3.3. At this pH the osmotic pressure of a gelatin solution is nearly a maximum. A 1 per cent solution of gelatin chloride has an osmotic pressure of about 450 mm. water at pH 3.4. Only a small part of this osmotic pressure is due to the osmotic pressure of the protein particles themselves; the rest of the observed osmotic pressure of gelatin chloride solutions of pH 3.4 is due to the excess concentration of the crystalloidal ions inside the collodion bag (in which the osmotic pressure of the gelatin solution is measured) over that of the outside aqueous solution free from gelatin, and this quantity is determined by the Donnan equilibrium.<sup>4</sup>

Collodion bags of a content of about 50 cc. were filled with these suspensions and closed with rubber stoppers perforated with glass tubes serving as manometers to measure the osmotic pressure.<sup>4</sup>

<sup>3</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 232; *J. Gen. Physiol.*, 1921-22, iv, 97.

<sup>4</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 169; *J. Gen. Physiol.*, 1920-21, iii, 691.

The bags were put over night at 21°C. into beakers containing each 350 cc. of 0.001 N HCl in water. The next day the osmotic pressure was read, the P.D. between the gelatin chloride solution and the outside aqueous solution free from gelatin was measured (with a Compton electrometer and indifferent saturated KCl-calomel electrodes), and the pH inside and outside was determined with the hydrogen electrode. Table I gives the results of these observations.

TABLE I.

*Influence of Substitution of Powdered for Dissolved Gelatin on Osmotic Pressure and P. D*

Powdered gelatin per 100 cc., gm.....	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
Dissolved gelatin per 100 cc., gm.....	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Osmotic pressure.....	85	132	181	230	268	310	342	406	398
pH inside.....	3.16	3.20	3.18	3.19	3.22	3.27	3.28	3.30	3.33
pH outside.....	2.82	2.85	2.85	2.83	2.83	2.85	2.82	2.84	2.87
pH inside minus pH outside...	0.34	0.35	0.33	0.36	0.39	0.42	0.46	0.46	0.46
P.D. calculated, millivolts.....	20.0	20.5	19.0	21.0	22.5	24.5	26.5	26.5	26.5
Membrane potentials observed, millivolts .....	Between 23.0 and 18.0	Between 22.0 and 18.0.	23.0	21.0	22.5	25.0	26.0	26.5	27.0
	No constant reading.								

The table confirms the writer's former observation that the osmotic pressure of the gelatin solution diminishes the more the more of the dissolved gelatin is replaced by powdered gelatin. The latter obviously does not participate in the osmotic pressure.

The table shows furthermore that the P.D. observed at equilibrium between the gelatin solution and the outside aqueous solution varies much less than the osmotic pressure. It became necessary to ascertain whether or not this P.D. across the membrane which was measured with the aid of two indifferent electrodes (saturated KCl-calomel solution) was actually determined by the difference in the

hydrogen ion concentrations inside and outside, as we should expect if the membrane potentials are due to a membrane equilibrium. The pH inside and outside was therefore measured with the aid of the hydrogen electrode and the value 58 (pH inside minus pH outside) millivolts is called the calculated P.D. The reader will notice that the difference between the observed membrane potential (measured with indifferent electrodes) and the calculated P.D. is not more than 0.5 millivolt. This leaves no doubt that the observed P.D. is determined by the difference in the hydrogen ion concentration on the opposite sides of the collodion membrane and that this P.D. obeys Donnan's equilibrium equation.<sup>5</sup>

These facts then show that the protein aggregates participate in the Donnan equilibrium almost to the same extent as do the isolated molecules or ions of gelatin, and this participation finds expression in the fact that the membrane potentials are lowered comparatively little when dissolved gelatin is replaced by powdered gelatin. The same particles, however, do not contribute to the osmotic pressure for the reason that their share in the excess of chlorine ions is contained inside the solid particles, where it serves to increase the swelling of the particles. The swelling of solid protein particles is, as Procter and Wilson have shown,<sup>6</sup> due to the increase of osmotic pressure inside the particles caused by the Donnan equilibrium. In our experiment there exists inside of each particle of powdered gelatin a Donnan equilibrium whereby the concentration of Cl ions inside is greater than outside and this causes an osmotic pressure. Water will, therefore, diffuse into each granule until the cohesion pressure of the solid particles of gelatin equals the osmotic pressure inside the particles due to the Donnan equilibrium, and the particles will swell. When we therefore have a mixture of dissolved gelatin and powdered particles (micellæ) we have two different osmotic pressures; namely, first, the osmotic pressure of the gelatin in true solution, and, second, the osmotic pressure inside each solid particle of gelatin. The former is measured by the hydrostatic pressure of the column of water required to equalize the rate of dif-

<sup>5</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 120; *J. Gen. Physiol.*, 1920-21, iii, 557, 667; 1921-22, iv, 351, 463.

<sup>6</sup> Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

fusion in opposite directions through the membrane. This is the osmotic pressure of the protein solution in Table I. The osmotic pressure inside each particle of solid powdered gelatin results in swelling, *i.e.* in an increase of the force of cohesion between the molecules of the gel particle, and this effect does not appear in the osmotic pressure of the solution. Only that part of the osmotic forces in a protein solution appears in the form of hydrostatic pressure which is directly or indirectly due to the isolated molecules of the protein; and this hydrostatic pressure is diminished when part of the protein in solution is replaced by aggregates or micellæ of protein.

## II.

When a solution of gelatin chloride containing solid granules of gelatin is separated by a collodion membrane from an aqueous solution (free from protein) two different equilibria are established; one across the membrane between the aqueous solution outside and the gelatin solution *inside* the membrane, and a second one between the solid granules of gelatin and the gelatin solution in which the granules are suspended. At first thought it might seem strange that when solid granules of isoelectric gelatin are suspended in a solution of gelatin and HCl, there should arise a difference in the distribution of the H and Cl ions inside the solid granules and the surrounding gelatin solution. Yet this is the case, as Table II shows, and the reason is easily understood. In the solid granules of gelatin the concentration of protein molecules is much higher than in the weak solutions of gelatin surrounding the granules, and if HCl is added the concentration of gelatin ions must be higher inside the solid gelatin granules than in the dilute gelatin solution in which the granules are suspended. It follows from Donnan's theory that this difference in the concentration of protein ions inside the powdered particles and the solution must give rise to a Donnan equilibrium; as a consequence of which a P.D. must exist between the solid particles and the weaker gelatin solution.<sup>7</sup>

This consequence of the theory was confirmed by the following experiment. Mixtures of a solution of isoelectric gelatin and

<sup>7</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 145.

powdered isoelectric gelatin were made so that 100 cc. always contained 0.8 gm. of isoelectric gelatin in all. The proportion of solid and liquid gelatin varied, however, in each case as indicated in Table II. In each 100 cc. of the mixture were contained 8 cc. of 0.1 *N* HCl. The mixtures were kept for 2 hours at 20°C. and frequently agitated to accelerate establishment of equilibrium between granules and solution. The solid powdered gelatin was then separated from the supernatant liquid by filtration.

TABLE II.

*Donnan Equilibrium between Particles of Powdered Gelatin and Gelatin in Solution.*

Powdered gelatin per 100 cc., gm.....	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Dissolved gelatin per 100 cc., gm.....	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
pH of powdered gelatin.....		3.30	3.26	3.28	3.24	3.28	3.24	3.30	3.26
pH of supernatant gelatin solution.....	2.99	2.97	2.90	2.88	2.83	2.77	2.72	2.69	2.62
pH solid minus pH liquid gelatin.....		0.33	0.36	0.40	0.41	0.51	0.52	0.61	0.64
P.D. calculated, millivolts.....		19.0	21.0	23.0	24.0	29.5	30.0	35.5	37.0
P.D. observed, millivolts.....		14.5	17.0	17.0	17.5	23.0	26.0	30.0	33.0

The solid gelatin was melted and poured into the vessels described on page 154 of a recent book by the writer, and the P.D. between the solid and liquid gelatin was measured. The values are found in Table II, showing that there exists a considerable P.D. between the gelatin granules and weak solutions of gelatin, and that this P.D. increases with the relative increase in the concentration of solid gelatin, as was to be expected.

Measurements of the pH in the solid and liquid gelatin showed that this P.D. was determined by the Donnan equilibrium.<sup>8</sup> There exists, however, a discrepancy between calculated and observed values for the P.D., which requires further investigation. On the whole, however, the figures seem to prove that when a suspension of powdered protein in a weak gelatin solution is put inside a collodion bag, the latter dipped into an aqueous solution free from gelatin, two equilibria are established; namely, one between the solid

<sup>8</sup> The measurements of the P.D. between solid gelatin and solution are not as accurate as the measurements between liquids across a membrane.

gelatin and the solution of gelatin inside the collodion bag, and a second one between the gelatin solution inside the collodion bag and the outside aqueous solution free from gelatin.

This explains why the powdered particles of protein contained in a gelatin solution participate in the Donnan equilibrium and the membrane potential of the solution without adding to the osmotic pressure of the solution as measured by the hydrostatic pressure required to equalize the rate of diffusion of water in opposite directions through the membrane. The participation of the solid particles in the Donnan equilibrium leads to an osmotic pressure inside of each solid granule, but this osmotic pressure is measured in terms of cohesion pressure of the swollen particles.

This swelling of the solid particles increases the viscosity of the solution, and the writer has shown that this fact is a further support of the explanation of colloidal behavior on the basis of the theory of membrane equilibria.<sup>3</sup>

#### SUMMARY AND CONCLUSIONS.

1. It is shown that when part of the gelatin in a solution of gelatin chloride is replaced by particles of powdered gelatin (without change of pH) the membrane potential of the solution is influenced comparatively little.

2. A measurement of the hydrogen ion concentration of the gelatin chloride solution and the outside aqueous solution with which the gelatin solution is in osmotic equilibrium, shows that the membrane potential can be calculated from this difference of hydrogen ion concentration with an accuracy of half a millivolt. This proves that the membrane potential is due to the establishment of a membrane equilibrium and that the powdered particles participate in this membrane equilibrium.

3. It is shown that a Donnan equilibrium is established between powdered particles of gelatin chloride and not too strong a solution of gelatin chloride. This is due to the fact that the powdered gelatin particles may be considered as a solid solution of gelatin with a higher concentration than that of the weak gelatin solution in which they are suspended. It follows from the theory of membrane

equilibria that this difference in concentration of protein ions must give rise to potential differences between the solid particles and the weaker gelatin solution.

4. The writer had shown previously that when the gelatin in a solution of gelatin chloride is replaced by powdered gelatin (without a change in pH), the osmotic pressure of the solution is lowered the more the more dissolved gelatin is replaced by powdered gelatin. It is therefore obvious that the powdered particles of gelatin do not participate in the osmotic pressure of the solution in spite of the fact that they participate in the establishment of the Donnan equilibrium and in the membrane potentials.

5. This paradoxical phenomenon finds its explanation in the fact that as a consequence of the participation of each particle in the Donnan equilibrium, a special osmotic pressure is set up in each individual particle of powdered gelatin which leads to a swelling of that particle, and this osmotic pressure is measured by the increase in the cohesion pressure of the powdered particles required to balance the osmotic pressure inside each particle.

6. In a mixture of protein in solution and powdered protein (or protein micellæ) we have therefore two kinds of osmotic pressure, the hydrostatic pressure of the protein which is in true solution, and the cohesion pressure of the aggregates. Since only the former is noticeable in the hydrostatic pressure which serves as a measure of the osmotic pressure of a solution, it is clear why the osmotic pressure of a protein solution must be diminished when part of the protein in true solution is replaced by aggregates.



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